# Parasite Factors, Prognostic Markers and Epidemiological Aspects in *Babesia canis* Babesiosis

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# Parasite Factors, Prognostic Markers and Epidemiological Aspects in *Babesia canis* Babesiosis

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# PART I SUMMARY

## 1. Summary

The triad of the phylogenetically related protozoan blood parasites *Babesia*, *Plasmodium* and *Theileria* are the causative agents of important human and veterinary diseases worldwide. Canine babesiosis, a persistent endemic disease in diverse dog populations, is caused by apicomplexan hemoparasites of the genus *Babesia*. Parasite host transmission is mediated by ticks and is facilitated by the international and domestic transportation of dogs as well as the focal availability of tick vectors. The presence of chronic subclinical carrier states in dogs, the inability to completely eliminate all infections, and the transovarial transmission of the parasite within the vector tick population further lead to a spreading of new infections.

*Babesia canis* is the predominant and clinically relevant canine *Babesia* species in Europe. Although the pathophysiology of canine babesiosis has been extensively studied, parasite-host interactions of the parasite are poorly understood. In common, the peripheral parasitemia remains usually low which however does not prevent severe clinical signs and high fatality rates. Hence, the pathogenic capacities of *B. canis* lie within the secreted factors, all of which have been poorly characterized. This PhD project aimed to characterize key factors involved in *B. canis* infections, such as the *B. canis* secretome which affects infected erythrocyte biology (Subproject 1), the description of clinical factors and pathophysiologic mechanisms of babesiosis in dogs (Subproject 2), and the analysis of tick-vector dissemination after a locally restricted outbreak of *B. canis* (Subproject 3).

Like other apicomplexan parasites, *B. canis* has developed a repertoire of organelles, pathways and strategies to survive, subsist and replicate within the infected host-cell. In a first subproject, we assembled and annotated the *B. canis* reference genome from a virulent Hungarian parasite strain to generate an understanding of *B. canis* virulence and pathogenicity factors. Out of the 3467 predicted gene models we identified 509 potential secreted parasite proteins. To identify specific parasite protein candidates involved during acute crisis of the disease, we analyzed the *B. canis* blood stage proteome and transcriptome using parasites isolated from experimentally infected dogs. Our data indicate the presence of parasite factors involved in cytoadherence, immune evasion, and infected red blood cell remodeling. These combined analyses provide a predicted and partially validated set of exported factors from *B. canis* blood stages potentially involved in the direct parasite-host interplay during the acute phase of the disease.

In a second subproject, we focused on host factors during acute *B. canis* infections which are associated with severe and fatal clinical outcomes. By examining 28 different laboratory parameters in 15 naturally infected animals at the time of first admission and before any treatment on the one hand, and in the course of 3 experimentally infected dogs on the other hand, we uncovered 7 prognostic markers associated with poor outcomes. Severe clinical outcomes were associated with

moderate anemia, severe thrombocytopenia, mild to moderate leukopenia, hyperlactatemia, moderately increased serum phosphate and triglyceride concentrations, and moderately decreased total serum protein concentrations. However, prognosis is not correlated to clinical signs or the level of parasitemia which remains usually low. Hence, altered laboratory parameters could be attributed to an excessive host immune-response contributed directly by parasite induced septic conditions.

In a third subproject, we investigated the occurrence of the vector tick following a recent outbreak of *B. canis* babesiosis in northeastern Switzerland. Data about tick species infesting dogs and cats are generally rare. Changes in the endemic foci of tick populations and invasions of tick species into new areas have become evident in Europe, leading to continuous changes in the epidemiology of tick-transmitted diseases. By a novel local pet-owner assisted sampling strategy we investigated the occurrence of different tick species on dogs and cats and focused on the presence of *D. reticulatus*, which is the tick vector for *B. canis* babesiosis. We identified *Ixodes ricinus* as the most abundant species on both hosts, followed by *I. hexagonus* and *D. reticulatus* both with prevalences below 2%. The local occurrence of *D. reticulatus* was confirmed by collecting ticks in a marsh-like environment with open meadows, loose trees and intense solar radiation. Our sampling strategy allowed to identify for the first time the vole or shrew tick *I. trianguliceps* on cats. Our survey demonstrated that pet owners can assist in collecting important epidemiological data on local tick species and contribute to a cost-effective tick surveillance, thus laying the groundwork for the investigation of local tick-transmitted diseases.

All these data demonstrate the urgent need for novel intervention approaches such as improved diagnostic tools and treatment options for canine babesiosis and the development of vaccine strategies in order to effectively contain the infection. This PhD thesis was partially funded by the “Forschungskredit” of the University of Zurich (grant nos. 55080506 and FK13-053).

## 2. Zusammenfassung

Die drei phylogenetisch verwandten Blutprotozoa der Gattung *Babesia, Plasmodium*, und *Theileria* sind Verursacher bedeutender human- und veterinärmedizinischer Erkrankungen. Die canine Babesiose, eine persistierende endemische Erkrankung in diversen Hundepopulationen, wird durch Blutparasiten der Gattung *Babesia* verursacht. Der Parasit wird durch Zecken übertragen, und seine Verbreitung wird durch den internationalen und innerstaatlichen Transport von Hunden sowie durch das lokale Vorkommen der Vektorzecken vermittelt. Chronisch subklinische Träger, die folglich erschwerte Elimination aller Infektionen, und eine transovarielle Übertragung in der Vektorzecke fördern zusätzlich die Verbreitung von neuen Infektionen.

*Babesia canis* ist die vorherrschende und klinisch relevante canine *Babesia*-Art in Europa. Obwohl die Pathophysiologie der caninen Babesiose umfangreich erforscht wurde, sind die Interaktionen zwischen Parasit und Wirt kaum bekannt. Im Allgemeinen bleibt die periphere Parasitämie tief, was jedoch schwere klinische Verläufe und hohe Sterblichkeitsraten nicht verhindert. Die pathogenen Eigenschaften von *B. canis* werden durch sekretierte Faktoren vermittelt, welche bisher kaum charakterisiert wurden. Ziel dieser Doktorarbeit ist die Identifizierung von Schlüsselfaktoren der akuten *B. canis*-Infektion, wie etwa das *B. canis*-Sekretom welches die Biologie von infizierten Erythrozyten beeinflusst (Teilprojekt 1), die Beschreibung von klinischen Faktoren und pathophysiologischen Mechanismen der Babesiose bei Hunden (Teilprojekt 2), und die Untersuchung der Verbreitung von Vektorzecken bei einem lokal begrenzten Ausbruch von *B. canis* (Teilprojekt 3).

Wie andere Apicomplexa verfügt *B. canis* über ein Repertoire von Organellen, ''Pathways'' und Strategien, welche sein Überleben in der infizierten Wirtszelle sichern und eine Vermehrung ermöglichen. In einem ersten Teilprojekt haben wir für ein besseres Verständnis von spezifischen Virulenz- und Pathogenitätsfaktoren das Genom eines virulenten ungarischen *B. canis*-Stammes sequenziert und annotiert. Von den 3467 prognostizierten Genmodellen identifizierten wir 509 potentielle vom Parasiten sekretierte Proteine. Um eine Reihe von Parasiten-spezifischen Proteinkandidaten zu identifizieren welche während der akuten Phase der Erkrankung mitwirken, analysierten wird das Proteom und Transkriptom von *B. canis*-Blutstadien in experimentell infizierten Hunden. Unsere Daten deuten auf die Existenz von parasitären Proteinen hin, welche bei der Zelladhärenz, Immunevasion und der Umformung der Wirtszelle eine Rolle spielen. Die Kombination unserer Analysen liefert eine Liste mit prognostizierten und partiell bestätigten exportierten Faktoren von *B. canis*-Blutstadien, die wahrscheinlich direkt an der Parasit-Wirt-Interaktion während der akuten Krankheitsphase beteiligt sind.

In einem zweiten Teilprojekt fokussierten wir auf Wirtsfaktoren während der akuten *B. canis*-Infektion, die mit schwerwiegendem und fatalem klinischen Ausgang assoziiert sind. Wir untersuchten 28 verschiedene Laborparameter bei 15 natürlich erkrankten Hunden bei deren ersten Vorstellung und

vor jeglicher Behandlung, sowie bei 3 experimentell infizierten Hunden während deren Krankheitsverlauf. Dabei identifizierten wir 7 prognostische Marker, welche mit schweren Krankheitsverläufen assoziiert scheinen. Letztere beinhalten im Allgemeinen eine moderate Anämie, schwerwiegende Thrombozytopenie, milde bis moderate Leukopenie, Hyperlaktatämie, moderat erhöhtes Serum-Phosphat und erhöhte Triglyzerid-Konzentrationen. Allerdings korreliert die Prognose nicht mit klinischen Symptomen oder der Höhe der Parasitämie, welche meist tief bleibt. Die veränderten Laborparameter lassen sich nur durch eine übermässige Wirts-Immunantwort mit einer Beteiligung von parasiten-induzierten septischen Konditionen erklären.

In einem dritten Teilprojekt haben wir nach einem Ausbruch einer *B. canis*-Babesiose im Nordosten der Schweiz das Vorkommen der Vektorzecke untersucht. Daten über den Zeckenbefall bei Hunden und Katzen sind generell rar. Veränderungen in der lokalen Verbreitung von Zeckenpopulationen und die stetige Invasion von Zeckenarten in neue Gebiete gewinnt in Europa zunehmend an Wichtigkeit und führt zur stetigen Veränderung der Epidemiologie von Zecken-übertragenen Krankheiten. Mit einer neuen Beprobungsstrategie in Zusammenarbeit mit Tierbesitzern untersuchten wir das lokale Vorkommen verschiedener Zeckenarten auf Hunden und Katzen und legten spezielles Augenmerk auf *Dermacentor reticulatus*, den Vektor der *B. canis*-Babesiose. Wir identifizierten *Ixodes ricinus* als die häufigste Spezies bei beiden Tierarten, gefolgt von I. hexagonus und der *B. canis*-Vektorzecke *D. reticulatus* mit Prävalenzen unter 2%. Das lokale Vorkommen von *D. reticulatus* wurde durch flaggen von Zecken in einer moorartigen Umgebung mit offenen Auen, wenigen Bäumen und intensiver Sonneneinstrahlung bestätigt. Unsere Beprobungsstrategie führte zudem zum erstmaligen Nachweis der Mauszecke *I. trianguliceps* auf Katzen. Unsere Strategie zeigt auf, dass Tierbesitzer zur Erhebung wichtiger epidemiologischer Daten und einer kosteneffizienten Zeckenüberwachung beitragen können, und erschafft damit die Basis zur Erforschung lokaler Zecken-übertragener Krankheiten.

All diese Daten zeigen den dringenden Bedarf an neuen Interventionsansätzen, wie z.B. die Optimierung diagnostischer Werkzeuge, die Verbesserung der Behandlungmöglichkeiten oder die Entwicklung von Vakzinestrategien auf, um eine effektive Eindämmung der caninen Babesiose zu erreichen. Diese Doktorarbeit wurde teilweise durch den Forschungskredit der Universität Zürich finanziert (Projekt-Nr. 55080506 und FK13-053).

# PART II INTRODUCTION

## 1. The biology of *Babesia canis*

### 1.1. *Babesia* and the phylum Apicomplexa

Babesiosis is caused by an intra-erythrocytic apicomplexan parasite of the genus *Babesia* which is transmitted by ticks. The name honors the Romanian bacteriologist Victor Babes who first described a hemolytic disease in sheep and cattle in 1888. Few years later in 1893, the parasitic agent and the transmission cycle was discovered by Theobald Smith and Fred Kilborne.[1,2] Furthermore, they introduced the epidemiological concept of arthropods serving as disease vectors.[3] Today, over 100 *Babesia*-species have been described, some of which can cause important diseases in domestic animals, including pigs, horses, cattle, sheep, goats, cats, and dogs.[4] Most species parasitize rodents, farm animals, and carnivores.

The phylum of Apicomplexa is composed of a large group of protozoan parasitic organisms, consisting of over 5000 species, and a myriad of unnamed species are estimated to exist.[5,6] The Apicomplexa are defined by the presence of a conserved apical complex consisting of secretory organelles named micronemes and rhoptries and a set of spirally arranged microtubules and conoids in some species. Further secretory organelles are present in these protists called dense granules or spherical body in the piroplasmids.[7,8] Apicomplexan parasites infect a wide range of organisms from mollusks to mammals.[9] They have a significant economical and medical impact by causing diseases affecting veterinary and human medicine. Important diseases include, amongst many others, babesiosis (*Babesia* spp.) in animals and humans, theileriosis (*Theileria* spp.) in animals, malaria (*Plasmodium* spp.) in humans, and coccidiosis in various forms by *Cryptosporidium* spp., *Isospora* spp., *Eimeria* spp., *Sarcocystis* spp., *Toxoplasma gondii*, and *Neospora caninum*.

There is some discussion about phylogenetic relationships within Apicomplexa (Figure II-1). Together with dinoflagellates and the ciliates, the apicomplexans are classified as Alveolata.[10,11] Based on phenotypic characteristics and organism lifestyle, the Apicomplexa is traditionally arranged in four groups: the coccidians, the gregarines, the haemosporidians and the piroplasmids.[6] Nevertheless, the evolutionary relationships among the four groups and their species are presently unclear and do not account modern molecular data.[11,12] Besides these groups, there are many small outgroups and species forming independent trees, for example the cryptosporidia (which are sometimes classified to a separate group of 'cryptosporidia' or 'eimeriorines'). On the other hand, new species were reclassified to Apicomplexa, like *Nephromyces*, an endosymbiotic marine protist.[13] This organism was recently classified to the haematozoa (syn. Aconoidasida[12]), to which the order of haemosporidia and piroplasms belongs. All these examples show the diversity of the phylum of Apicomplexa.

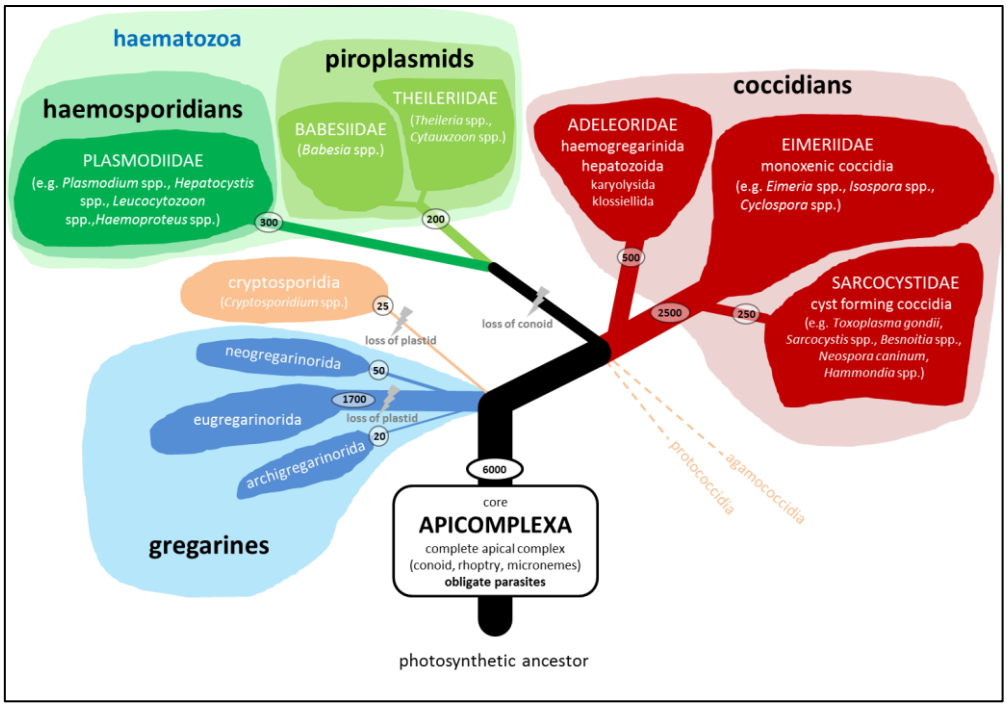


**Figure II-1. Tree of Apicomplexa** (adapted and modified [11,14-16]). Four traditionally arranged parasitic groups are colored differentially (gregarianes, coccidians, haemosporidians, and piroplasmids). Outlying groups, like cryptosporidia, which likely emerged from within gregarines, are marked separately. Thickness of branches indicates diversity and numbers give a rough estimate of named species.

## 1.2. *Babesia* in dogs; development and epidemiology of canine babesiosis

Canine babesiosis is a clinically significant and geographically widespread hemo-protozoan disease of domesticated dogs and wild canids. Like most members of Apicomplexa, *Babesia* species undergo a complex lifecycle, involving asexual and sexual reproduction (Figure II-2).

Once in the host's bloodstream, the parasite invades, feeds and replicates within infected red blood cells (iRBC) during repeated phases of asexual reproduction (merogony). Reproduction happens within few hours after invasion. Lysis of iRBC releases the parasites (merozoites) into the circulation which rapidly re-invade red blood cells (RBC). RBC invasion by *Babesia* is similar to that of many other apicomplexan parasites including related human pathogens *Plasmodium* and *Toxoplasma*, with numerous proteins on the parasite surfaces and within apical secretory organelles mediating the complex multi-step invasion process.[7] Unlike other apicomplexan parasites such as the close relative *Plasmodium*, intracellular *Babesia* stages does not reside in a parasitophorous vacuole (PV), which in fact disappears within minutes after RBC invasion and leave the parasite directly in the host cell cytoplasm.[17] When the parasites enter a susceptible tick upon a blood meal, parasites undergo sexual reproduction (gamogony), followed by multiple rounds of asexual replication (first schizogony and then, sporogony), resulting in numerous sporozoites (in the tick salivary glands) which are infective for the next canid host.

*Babesia* species are broadly divided into two phenotypic groups (large and small *Babesia*), based on the relative sizes of the merozoites (3-5 µm for large and 1.5-2.5 µm respectively). Merozoites appear round to pear-shaped under the light microscopy (lending piroplasms its name). The different species which have been reported in dogs are found in different tick-vector species prevalent in different regions (Table II-1). *B. canis* is the predominant and clinical relevant canine *Babesia* species in Europe. The geographical distribution of *B. canis* in Europe is largely dependent on the habitat of the vector tick species *Dermacentor reticulatus*. Canine babesiosis in Europe was described for the first time from Piana and Galli-Valerio in Italy in 1895.[18] Since then, *B. canis* has spread northwards and local transmission could be documented in countries like the Netherlands, Norway and Lithuania.[19-21] An overall presence of canine babesiosis and local differences in the occurrence have been described from Spain, Portugal, France, Poland, and Hungary, which are known to be endemic countries, with hyper-endemic areas in local parts of the countries.[22-24] Furthermore, babesiosis is endemic in parts of Germany with a proceeding expansion to new areas.[25-27] Contrary to the endemic transmission, in other countries the disease is considered as exotic and known as a travel-disease like in the Benelux countries, Austria, and Switzerland where low prevalences and focal occurrence are related either to imported cases or to small autochthonous foci of *B. canis* infections.[27-29]

Table II-1. Characteristics of confirmed canine *Babesia* species

| | **Species** | **Vector** | **Geographic distribution** | **Comments and recent references** |
|---|---|---|---|---|
| **small *Babesia* sp.** | *Babesia gibsoni* | *Haemaphysalis longicornis*<br>*H. bispinosa*<br>*Rhipicephalus sanguineus* (?) | Asia, Eastern Europe, emerging worldwide | |
| | *B. conradae* | *R. sanguineus* (?) | USA (California) | Closely related to piroplasms recovered from ungulates and humans [30] |
| | *B. annae* (syn. *Theileria annae*, *B. vulpes, B. microti*-like, Spanish dog isolate) | *I. hexagonus* (?) | Immunocompromised dogs from northern Spain, eastern Canada, North America | Parasite was detected in foxes in Europe, North America, Canada [31-34] |
| **large *Babesia* sp.** | *B. canis* | *Dermacentor spp.* | Europe | Emerging disease in northern and eastern countries |
| | *B. vogeli* | *R. sanguineus* | Tropical and subtropical regions, around Mediterranean basin | |
| | *B. rossi* | *H. laechii* | Sub-Saharan Africa and Southern Africa | |
| | *Babesia* sp. (North Carolina Isolate) | ? | East and southeast United State | Immunocompromised dogs [35,36] |

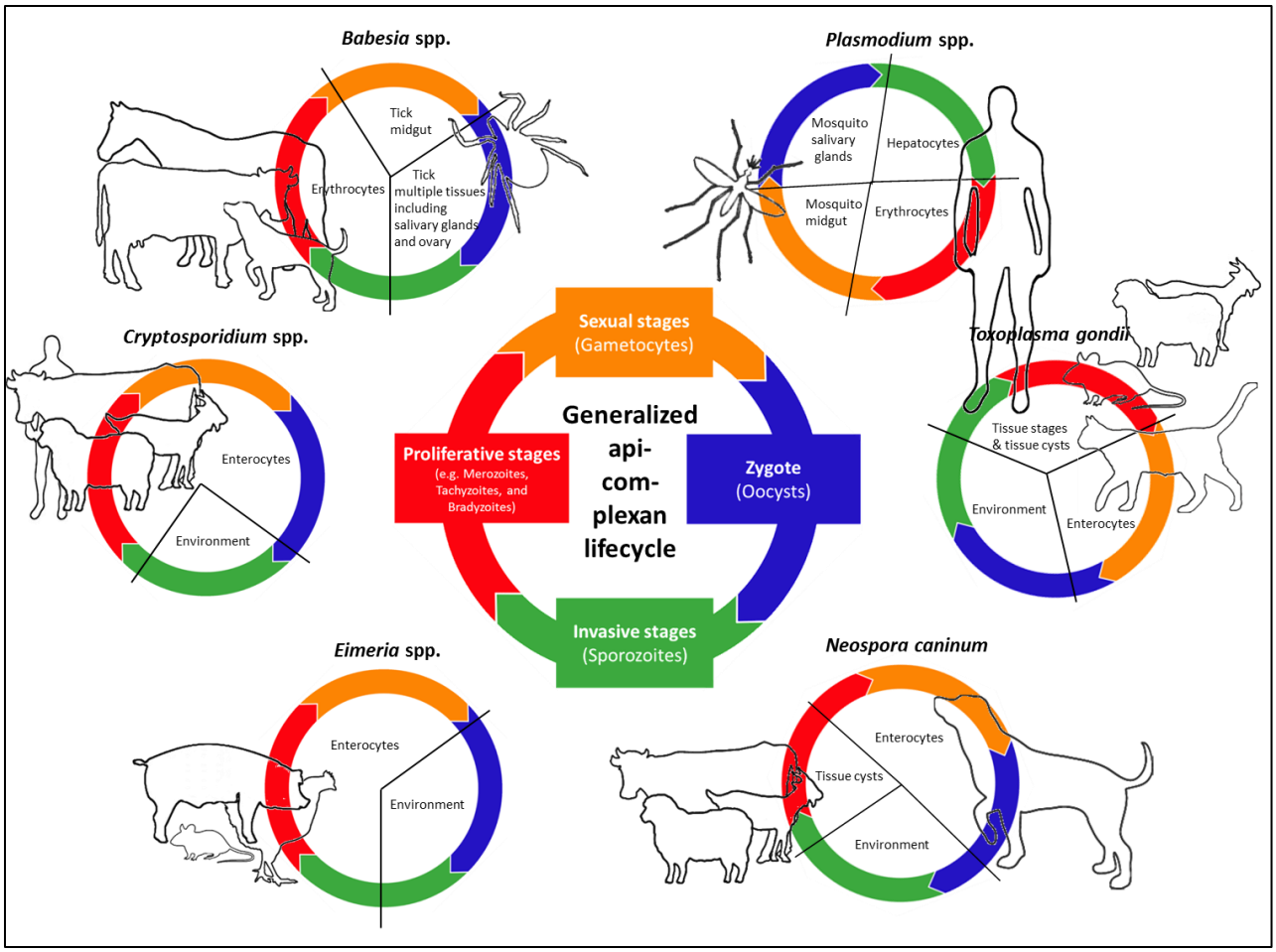


**Figure II-2. General life-cycles of selected apicomplexan species.** Simplified lifecycles of apicomplexan parasites are pointed out to highlight the differences in host- and cell type specificity. A selection of important hosts is illustrated. (Adapted and modified from Heitlinger *et al.*[37])

## 1.3. Pathophysiology of *B. canis* infections.

Following an incubation period of 7 to 21 days after tick exposure, immunologically naïve dogs mainly become acutely infected (rarely chronic or per-acute).[16,38] In dogs, *B. canis* cause highly variable clinical outcomes from subclinical infections to severe and fatal disease, characterized by hemolytic anemia (pale mucous membranes), lethargy, weakness, pigmenturia (dark discoloration of the urine), fever, anorexia, vomiting and jaundice.[4,39] Although the peripheral parasitemia remains usually low (<1% of iRBC), especially the per-acute to acute disease represents an emergency situation with high fatality rates.[40-44] Based on clinical signs and the outcome of the *B. canis* infection, at least two different groups (sometimes designated as different *B. canis* strains) have been suggested.[44,45] (1) *B. canis* strains inducing often complicated forms, with a high mortality rate (12–20%) in Central- and Eastern Europe (mainly Hungary and Croatia [42,44] and (2) *B. canis* infections showing mainly uncomplicated forms with low mortality rates (<5%, the lowest being in France around 1%) in other countries such as Italy, Spain,

Portugal, and France.[19,22,41,46-50] Such differences in the virulence of *B. canis* strains strongly suggests genetic heterogeneity among *B. canis* strains (see chapter 1.4.).

The pathophysiology of *B. canis* babesiosis is multifactorial and dependent on the immune-response and age of the host, as well as the involved parasite strain (origin of infection). Diverse underlying mechanisms have been discussed to be involved in the *B. canis* pathophysiology, inducing different observable clinical syndromes:

(1) Anemia is a common observation and the predominant clinical syndrome associated with canine babesiosis characterized by intravascular and extravascular hemolysis. The parasitemia does not correlate to the proportion of RBC loss. Therefore, additional mechanisms for RBC destruction other than mechanical lysis by the release of merozoites have been proposed including immune-mediated (immune-mediated hemolytic anemia and cytokine reactions), parasite-induced by toxic hemolytic factors, and by oxidative stress response.[51-54]

(2) Host inflammatory responses (and associated clinical signs) have been shown to be crucial in the general pathophysiological mechanisms that underlies babesiosis.[55,56] This includes oxidative stress with lipid peroxidation and the generation of methemoglobinemia, increased macrophage production of superoxide and other reactive oxygen species, acute phase response with RBC aggregation and the promotion of vascular stasis, leading to ischemia, thrombosis, and tissue hypoxia and thus, end-organ damage. Because the symptoms in babesiosis, like in human malaria caused by *P. falciparum*, can be classified as protozoal sepsis, it was suggested that the inflammatory mechanisms in this disease are similar to those of other septic conditions clinically characterized by systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS). Furthermore, documented complications include severe thrombocytopenia, disseminated intravascular coagulation (DIC), acute renal failure (ARF), hepatopathy, rhabdomyolysis, non-cardiogenic pulmonary edema, CNS dysfunction, pancreatitis, systemic hypotension, cardiac dysfunction, hypoglycemia, and metabolic acidosis with hyperlactatemia (reviewed in detail in [44,57]).

(3) Hypotensive shock is a frequent complication in canine babesiosis, and can occur at any point during the disease process.[58-60] Proposed mechanisms include parasite induced activation of potent vasodilators and hypotensors, such as bradykinin and kallikrein, and host complement system activation of C3a and C5a with resultant vasodilation (relative hypovolemia and drop in blood-pressure), production of vasoactive proteins (host and parasite), increased capillary permeability, decreased intravascular volume, and myocardial depression.[58] In turn, hypotension enhances parasite sequestration and hypoxia, leading to a vicious cycle of increased disease severity with perpetuated inflammation and hypotension.[16,61]

## 1.4. Diagnosis of *B. canis* infections

A tentative clinical diagnosis of acute babesiosis is based on the transmission risk, tick exposure and history of travel to an endemic region. However, an accurate clinical diagnosis is difficult because clinical signs are mainly unspecific. In acute infections, diagnosis is routinely based on the direct demonstration of parasites in Giemsa-stained blood smears allowing a prompt specific diagnosis by experienced investigators. In some cases however, morphological diagnosis is made difficult by low parasitemia which is commonly observed in subclinical-, latent-, but also in acute infections.[40, 41] In related canine *B. rossi* infections, it was demonstrated that the likelihood to detect parasites could be increased by capillary smears or buffy coat concentration techniques.[4, 62, 63] In order to increase sensitivity, detection of *B. canis* by PCR and other DNA based methods, such as loop mediated isothermal amplification (LAMP), have been reported.[64-67], increasing the detection limit more than hundred times [68] allowing to detect parasites even in small volumes with morphological untraceable low parasitemia.[69]

To demonstrate parasite exposure, serological antibody detection by immune fluorescence antibody test (IFAT) is considered the most specific methodology,[70] although the interpretation of the results is influenced by the subjective judgement of the operator. Hence, other antibody-detection assays have been evaluated, including slide ELISA, sandwich ELISA, competitive ELISA and dot- enzyme immunoassay with superior sensitivity but significant lower specificity.[71-74] By detecting antibodies, a high number of false-negative results was discussed to occur in very young or immunocompromised patients, or at acute disease onset, as sero-conversion usually takes 8 to 10 days.[75, 76] Limitations are also reported in endemic regions, where antibody reactions are not always associated with clinical disease.[77] Nevertheless, antibody detection assays are beneficial for documenting parasite exposure, especially if direct parasite detection fails, or for screening travelers. Furthermore, diagnosis could be confirmed by examining paired acute and convalescent sera. Some other techniques including flow cytometry based techniques have been developed for the diagnosis of *B. canis*.[54, 70]

## 1.5. Genetic characterization and proteomic background of *B. canis*

To date, large *Babesia* parasites that infect dogs are separated into the three genetically distinct species, *B. canis*, *B. rossi*, and *B. vogeli*. Whole genome assemblies of none of these species have been published. This is just one reasons why so far not many *B. canis* genes and proteins have been characterized and the genetic and proteomic context of parasite-host interactions is poorly understood.

PCR amplification of the 18S rRNA gene was used to demonstrate the diversity of *B. canis* strains in Poland,[49] although the 18S rRNA gene is less suited to study genetic diversity of a given species

because it is relatively conserved among strains of a given species.[64, 78-80] Genetic variability and antigenic variation are important mechanisms for the survival of *Babesia* parasites in their vertebrate hosts. By comparative analysis and hybridization approaches to the major antigenic adhesion protein from *B. divergens (Bd*37*)*, the *B. canis* Bc28 merozoite surface protein multi-gene family has been identified.[45, 81] Based on the polymorphism of the Bc28.1 gene, genetic diversity of different *B. canis* strains in Europe has been confirmed. Therefore, based on 18S rRNA gene two different groups were described (A and B), and three groups based on Bc28 gene (also A, B, and 34).[45] In conclusion, *B. canis* Bc28.1-B strains appear relatively virulent, whereas *B. can*is Bc28.1-A strains can be either relatively virulent if of the 18S rRNA-B genotype or mild if of the 18Sr RNA-A genotype, which is in agreement with their previous classification.[40, 82, 83]

Substantial progress in *B. canis* protein characterization origin from vaccine research. Since decades it is known that a crude soluble parasite antigen (SPA) from blood culture supernatant of several *Babesia* species can be used as a vaccine against the clinical manifestation of babesiosis challenged with the same parasite strain.[84-86] Furthermore, classical historical experiments showed that the plasma of infected animals can protect healthy animals from a challenge infection.[87] Proteins from the SPA fraction containing also glycoproteins are highly immunogenic.[88, 89] Nevertheless, the composition of secreted *B. canis* proteins in the supernatants of blood cultures is largely unknown.

On the proteomic level, Bc28.1 and Bc28.2 proteins from the Bc28 family are some of the few characterized proteins in *B. canis*. The small RBC binding protein Bc28.1 is a major antigen on the surface of *B. canis* merozoites. The gene encodes a 28 kDa GPI-anchored protein. By antibody-detection approaches, a localization of the Bc28 proteins on the parasites surface, on the host cell membrane and secreted extracellular to the host could be demonstrated.[90] The function of Bc28.1 and Bc28.2, the only cloned and sequenced members of this family, are still unknown. It is proposed that these antigens play a critical role in the initial attachment of merozoites to the RBC membrane during the invasion process.[91] Comparison of the two genes (Bc28.1 and Bc28.2) and predicted amino acid sequences demonstrates highly conserved N-terminal regions, and C-terminal regions that are divergent. As a consequence, the GPI-anchor that is present in Bc28.1 is not predicted in Bc28.2.[81]

Recently, a complementary DNA expression library was used to screen antigens for novel sero-diagnostic techniques for the detection of *B. canis* infections.[92] This approach resulted in the description of a merozoite surface antigen (BcMSA1) and a secreted protein (BcSA1) as potential diagnostic antigen candidates. Polyclonal mouse sera raised against recombinant forms of these two

proteins and confocal microscopic observations with *B. canis* iRBC revealed that BcMSA1 and BcSA1 locate on the surface of the parasites, whereas BcSA1 is also located cytosolic.

By aiming to discover other vaccine candidates, an extrachromosomal dsRNA-encoded protein (Bcvir15) with putative functions in intracellular growth of the parasite has been described.[93] Whether this gene is derived from a virus or from other apicomplexan organelles harboring DNA (mitochondria, apicoplast) has not been further investigated to date.

## 2. Parasite-host interaction – insights from related apicomplexan organisms

How *Babesia,* which are tiny pathogens and should be kept in check by the host, manage to survive lies within a large number of secreted- and surface-associated factors that transform infected host cell, and that are major players in parasite-host interaction, virulence, and pathogenicity. In this respect, apicomplexan parasites have developed a repertoire of organelles, strategies and pathways. This includes factors secreted in a coordinated and sequential manner during the complex multi-step invasion process, modulated by specific secretory organelles (micronemes, rhoptries, and dense granules/spherical bodies) which are shared amongst apicomplexan parasites. This is well described in the ubiquitous zoonotic and well-studied apicomplexan *Toxoplasma gondii*,[94, 95] in *Plasmodium spp*.,[96, 97] and in bovine *Babesia* spp.[98-101] In order to translocate and communicate through different membranes (from parasite and host), intracellular parasites evolved the ability to remodel and modify the infected host cell. For example, *Plasmodium* spp. and *B. bovis* alter the infected host cell to generate an endothelial adhesive phenotype and sequester to escape degradation by the host defense system.[102-104] This active remodeling requires export of parasite proteins into the host cell cytoplasm and beyond with direct interaction with the host proteome.[105]

### 2.1. Secretory organelles in apicomplexan parasites

As a condition for adaption to diverse niches and hosts, apicomplexan parasites have evolved characteristic organelles and other ultrastructural features (see figure II-3). Secretory organelles consisting of micronemes, rhoptries and dense granules ('spherical bodies' in *Babesia*). Whereas the micronemes and rhoptries, both, are located and secrete their content at the apical tip of the parasite, the dense granules are not necessarily located apically and their proteins function in the PV, parasitophorous vacuolar membrane (PVM), or in tubular networks, depending on the stage and species.[106-108] Because of their specialized nature, the majority of proteins secreted from these organelles are unique to apicomplexan parasites and interesting targets for vaccine, drug and diagnostic development.[109]

### 2.1.1. Micronemes

The micronemes contain proteins (MICs) that are involved in the attachment of parasites to the host cell surface,[110] and in the formation of a connection to the host cell including the parasite actinomyosin motor,[111] which drives motility and invasion.[112] Several parasite adhesins that bind to the appropriate receptors on the host cell have been identified, which are highly diverse between different species due to co-evolution with different hosts and host cell receptors.[113, 114] Gliding of the parasite and movement through tissues is supported by proteases embedded in the basal end of the plasma membrane resulting in the shedding of the parasite ligands from the surface.[115, 116] Some microneme proteins are conserved between all apicomplexans, such as apical membrane antigens (like AMA-1 protein) that is thought to trigger further rhoptry release.[99, 117, 118] Notably, recent studies have highlighted the roles of MICs (TgMIC8 in *T. gondii*; PfEBA175 and PfAMA1 in *P. falciparum*) facilitating the secretion of rhoptry contents.[119-122] Besides invasion, a pore-forming protein released from the micronemes has been identified to be involved in host cell egress in *T. gondii* tachyzoites.[123]

### 2.1.2. Rhoptries

After invasion, rhoptry proteins are instrumental in the formation of the PVM.[124, 125] Different studies suggest that the rhoptry neck and rhoptry bulb are two distinct compartments, which contain different proteins, and are released differentially. For instance, the RON proteins stored in the rhoptry neck are critical for the formation of the tight junction between the parasite and the host cell, and therefore are important for host cell invasion. Rhoptry bulb proteins (ROPs) function predominantly downstream of invasion in modifying the vacuolar membrane and the host cell enhancing parasite survival within the cell.[7, 8, 109, 118, 126, 127] For example, some rhoptry kinases in *T. gondii*, which have been characterized in detail, are key modulators in virulence and host response.[128-130] Nevertheless, the biological functions of many rhoptry proteins remain largely unknown, owing to the lack of appropriate and robust methods to study essential parasite proteins although genetic manipulation strategies in cultivable species exist.[131, 132]

### 2.1.3. Dense granules / spherical bodies

The content of dense granules (spherical bodies in *Babesia*) is released immediately and constitutively after invasion.[8] The proteins of this organelle modify the host cell and structures to generate parasites own habitat.[7, 125, 133, 134] The spherical body proteins (SBP) found in *Babesia* and *Theileria* are considered to perform comparable functions to the dense granules found in other apicomplexan parasites. Nevertheless, no distinct function has yet been ascribed to any of these proteins. Over the last 20 years, four proteins have been identified which localize to these organelles termed SBP1, 2, 3, and 4.[135-138] Interestingly, none of the SBPs show any significant homology to any other known proteins.[137] One feasible possibility is that they are involved, either directly or indirectly,

in the formation of ridges on iRBCs, a function consistent with their demonstrated cellular localization at the iRBC membrane,[7] suggesting also an important role in parasite virulence.[139]

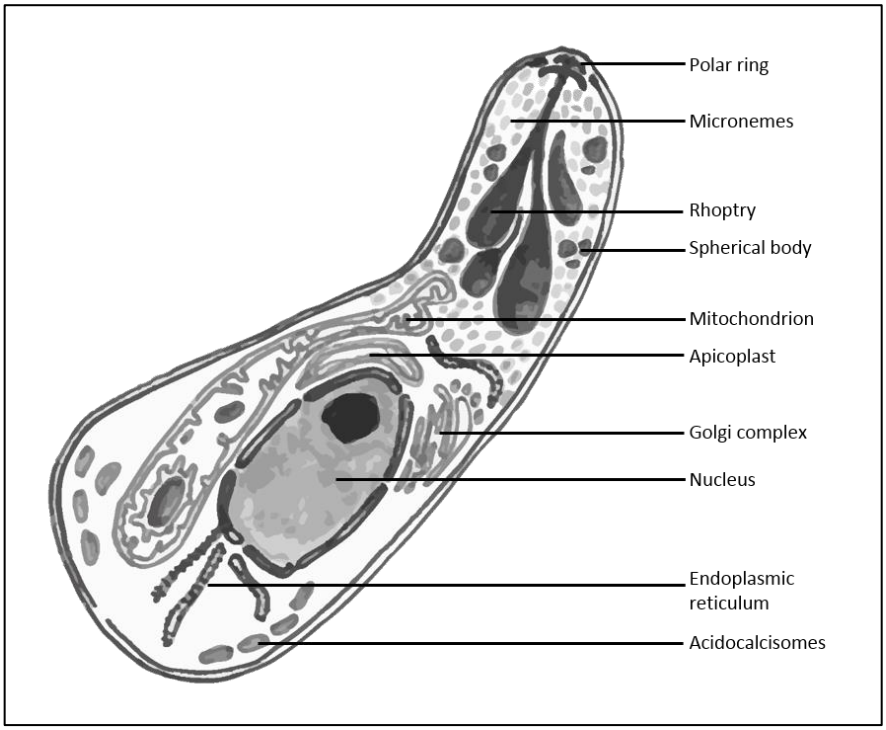


Figure II-3. Schematic picture of a *Babesia* merozoite.

## 2.2. Protein trafficking in the intra-erythrocytic apicomplexans *Plasmodium* and *Babesia*

Both *Babesia* and *Plasmodium* invade and replicate within RBCs. As mature RBCs lack any de novo protein synthesis and also have no trafficking pathways, the parasite has to bring along its own biochemical facilities. In *Plasmodium* it has been described that parasites induce new pathways that increase iRBC plasma membrane permeability to allow the uptake of nutrients and the removal of waste products, to avoid the host immune system and to escape destruction by the spleen.[99, 140-143] During the invasion process, the parasite forms a surrounding membrane in which it resides, the PVM. However, a fundamental difference between *Plasmodium* and *Babesia* is the maintenance of the PVM, which is present throughout the developmental cycle in *Plasmodium*, but in *Babesia* disappears within minutes after invasion leaving the parasite directly in the host cell cytoplasm.[17] Regardless of the fate of the PVM, the parasite has to have the capacity to secrete factors into the host cell to modify, create or modulate host cell functions, to suit the needs of the parasite. Hence, the parasite needs to create an export machinery.

In *Plasmodium*, a functional domain required for the export of parasite proteins into the host cell has been defined, termed *Plasmodium* host targeting (HT) motif or export element (PEXEL) for trafficking beyond the PVM.[144, 145] Furthermore, a second group of exported proteins does not contain

a PEXEL (so called PEXEL negative export protein, PNEP) but include other shared signals like essential transmembrane (TM) domains.[146, 147] Translocation of soluble PEXEL and PNEP proteins into the host cell is mediated via an ATP-driven translocon (*Plasmodium* translocon of exported proteins 'PTEX').[131, 147] Although a PVM translocon is a supposable gate for soluble proteins, it is unclear how TM proteins fit into this model.

PEXEL motifs were initially found to be absent in the predicted *B. bovis* exportome.[148] Comprehensive comparative *in silico* analysis of protein export in different apicomplexan species revealed PEXEL-like motifs (PLM) in *B. bovis* (but not in *Theileria parva*) involved in retaining proteins within spherical bodies and releasing their contents in a cell- cycle dependent manner.[149]

*Plasmodium* and *Babesia* both show expansion of large membrane-bound proteins transported to the cell surface: the variant PfEMP1 (erythrocyte membrane protein) in *P. falciparum* [150] and the variant VESA1 (variant erythrocyte surface antigen) in *Babesia*.[151] Members of both protein families share common features: many proteins lack classical N-terminal signal peptides for ER-targeting, but often contain internal TM domains. In both cases, orientation and mode of translocation remain unclear. Therefore, some kind of alternative non-classical secretion pathway could be present which yet has to be described.[147, 149]

### 2.3. Immune evasion and antigenic variation – strategies to escape host defense

In order to avoid splenic passage, some *Plasmodium* and *Babesia* species have evolved strategies for cytoadhesion and sequestration of iRBC to escape host immune defense, allowing to establish a persistent infection. This characteristic phenotype has been described in *P. falciparum*,[152] *P. chabaudi*,[153] *P. knowlesi*,[154] *B. bovis*,[151] *B. bigemina*,[155, 156] *B. canis* [157] and potentially *B. duncani*,[158] although the extent and structural basis likely differ among species. Furthermore, auto-agglutination of iRBC or agglutination to non-infected RBC (rosetting) have been observed.

Responsible for the adhesive phenotype in *P. falciparum* and *B. bovis* are mainly the immunovariant adhesins *P. falciparum* EMP1 (erythrocyte membrane protein 1) and the heterodimeric *B. bovis* VESA1 (variant erythrocyte surface antigen 1).[152, 159] Another benefit of the cytoadherence may also be a diminished access to circulating cells of the immune system.[160]

A close relationship of these variant adhesins to antigenic variation has been demonstrated.[161] Clonal antigenic variation of surface membrane proteins (i.e. to generate unique and changing B-cell epitopes) by protozoan parasites enables to avoid host immune responses and thus perpetuate long-term infections.

At the genetic level, the responsible genes in *P. falciparum* (e.g. var-genes) and *B. bovis* (ves1-genes) differ in structure and sequence. The increased genetic variation of the *B. bovis* ves1 gene family compared with the *P. falciparum* var gene family might result in a greater number of *B. bovis* strains

capable of evading the host immune system.[141, 162] Somatic recombination of gene segments from inactive ves genes into an actively transcribed locus allows the parasite to generate a potentially limitless repertoire of surface antigens, although overall evidence that every combination results in functional adhesive properties is missing.[163, 164]

In *P. falciparum*, additional surface proteins have been identified, including stevor, sequestrin, surfin, Pfmc-2TM, and the rifins.[141, 165] Further strategies of *Babesia* to escape host defense could be mediated by parasite proteins triggering anti-inflammatory cytokine release.[166] Altogether, *Babesia* parasites harbor an arsenal of factors for parasite-host interactions, which are far from being completely explored.

## 3. The framework of apicomplexan genomes

Genome assembly and annotation of intracellular apicomplexan parasites is challenging, because it is often difficult or impossible to generate appropriate genomic material by maintaining live and reproductive *in vitro* parasite cultures, in particular appropriate for *Babesia* species. Nevertheless, whole genome sequences of several parasites in the apicomplexan phylum have been published. This genomic information is predominantly collected and maintained by the EuPathDB (http://eupathdb.org) including 11 specialized resources for different apicomplexan species.[167]

The genome of several species in the triad of apicomplexan hemo-parasites (*Babesia*, *Plasmodium* and *Theileria*) have been made available. *P. falciparum* was the first apicomplexan genome to be sequenced,[168] followed by the sequencing efforts of many more species, including several other *Plasmodium*, *Babesia* and *Theileria* species (Table II-2).

Structurally, the genomes are organized in 4 to 14 linear chromosomes each with a centomeric and subtelomeric region, and telomeres. *Plasmodium* species have fourteen chromosomes of variable lengths.[168] On the other hand, *Theileria* sp. (*T. annulata* and *T. parva*) and *Babesia* sp. (*B. bovis* and *B. bigemina*) have four chromosomes.[164, 169-171] Genome features from available hematozoa are summarized in Table II-2. It is important to note that these genomes, although published, contain physical gaps and misannotated genes.[172] Most parasites in the apicomplexan lineage show two organelles with their own extrachromosomal DNA, the mitochondrion and the apicoplast. The apicoplast is an essential organelle in most apicomplexan (only in the genus *Cryptosporidium* an apicoplast is missing, which probably has been lost secondarily).[173] The functions of the apicoplast have not been exclusively figured out, but the apicoplast seems to play a role in fatty acid, isoprenoid, and iron-sulfur cluster synthesis, and co-opts with the mitochondrion in the heme synthesis and processing.[174-177]

Comparison and alignments of whole genomes reveal a high level of synteny within single hematozoan families (e.g. among *Plasmodium* species). Interestingly, extensive synteny was identified

between *Theileria* and *Babesia* species, accounting for shared phylogenies. Only limited orthology could be identified between *B. bovis* and *P. falciparum*, although there are shared clinical and pathological characteristics.[178, 179] (For graphical representation see Kissinger et al.[179])

A feature of parasite genomes is gene loss compared to their free-living relatives directly related to their parasitic lifestyle.[178, 180, 181] Hence, comparative functional analysis of apicomplexans proteomes shows many common features but also clear differences to free-living eukaryotes with similar genome size. There is a number of proteins that markedly differ, which are important for parasitic adaptations.[182] Therefore, these unique parasite genes which codes for various secreted, cell-surface, transmembrane and membrane-anchored proteins at the parasite-host interface are of special interest as ideal targets for new intervention approaches (like novel diagnostic tools, vaccines and therapeutics).

**Table II-2**. Summary of intra-erythrocytic apicomplexan genome sequence projects

| Organism | Genome size (Mbp) | Number of chromosomes | Number of predicted genes | G+C content (%) | Reference (year of first publication) |
|---|---|---|---|---|---|
| ***Plasmodium*** | | | | | |
| *P. berghei* ANKA | 18.78 | 14 | 5254 | 22.1 | (2005)[183-185] |
| *P. chabaudi chabaudi* | 18.97 | 14 | 5364 | 23.6 | (2005)[183, 185] |
| *P. coatneyi* Hackeri | 27.81 | - | - | - | (2014)[b] |
| *P. cynomolgi* strain B | 26.18 | 14 | 5776 | 40.4 | (2012)[186] |
| *P. falciparum* 3D7 | 23.33 | 14 | 5777 | 19.4 | (2002)[168] |
| *P. falciparum* IT | 22.98 | 14 | 5699 | - | (in preparation)[c] |
| *P. gallinaceum* 8A | 16.93 | - | - | - | (2006)[b] |
| *P. knowlesi* strain H | 24.40 | 14 | 5483 | 38.8 | (2008)[187] |
| *P. reichenowi* CDC | 23.92 | 14 | 6069 | 19.27 | (2014)[185] |
| *P. vivax* Sal-1 | 27.01 | 14 | 5626 | 42.3 | (2008)[188] |
| *P. yoelii yoelii* 17X | 22.76 | 14 | 6102 | - | (2014)[185] |
| *P. yoelii yoelii* 17XNL | 22.94 | - | 7774 | 22.6 | (2002)[189] |
| *P. yoelii yoelii* YM | 22.03 | 14 | 5833 | 21.1 | (2014)[185] |
| ***Babesia*** | | | | | |
| *B. bigemina* strain BOND | 13.84 | 4 | 5125 | 51 | (2014)[164] |
| *B. bovis* T2Bo | 8.18 | 4 | 3781 | 41.5 | (2007)[171] |
| *B. bovis* C9.1[a] | 7.61 | | 3726 | 42 | (2014)[164] |
| *B. microti* strain RI | 6.39 | 4 | 3554 | 36 | (2012)[190] |
| *B. divergens.* 1802A[a] | 9.58 | 4 | 4134 | 42 | (2014)[164] |
| *B. divergens* Rouen1987[a] | 8.97 | 4 | 4097 | 46 | (2014)[164] |
| ***Theileria*** | | | | | |
| *T. annulata* strain Ankara | 8.36 | 4 | 3845 | 32.5 | (2005)[170] |
| *T. equi* strain WA | 11.67 | 4 | 5397 | 39.5 | (2012)[191] |
| *T. orientalis* strain Shintoku | 9.01 | 4 | 4058 | - | (2012)[b] |
| *T. parva* strain Muguga | 8.35 | 4 | 4167 | 34.1 | (2005)[169] |
| ***Cytauxzoon*** | | | | | |
| *C. felis* strain Winnie | 9.11 | - | 4389 | 31.8 | (2013)[192] |

[a]data not available in EuPathDB

[b]data uploaded to EuPathDB (release 26). No publication available

[c]data available from Welcome Trust Sanger Institute. Manuscript in preparation

## 4. Aims of the thesis

Canine babesiosis is considered an emerging vector-borne disease, as numerous cases have been reported globally and in new areas throughout Europe including focal regions in Switzerland. Currently, host-parasite interactions in *B. canis* babesiosis are poorly understood. Hence, a significant improvement of our knowledge about the molecular details of the infection process and pathophysiology is required as a prerequisite for new intervention approaches.

Based on comparative data with related apicomplexan parasites we hypothesized that parasite-specific secreted factors are key determinants of pathogenicity inducing severe symptoms in infected dogs. Hence, the primary goal of my thesis was to identify and analyze the secreted *B. canis* proteome which affects iRBC biology and host response. In order to enhance our background knowledge about *B. canis* infection biology, objective was to investigate epidemiological aspects, to study clinical/pathological symptoms in canine babesiosis and to establish parasite genomic-, transcriptomic and proteomic resources. The project was funded by the "Forschungskredit" of the University of Zurich (grant nos. 55080506 and FK13-053) and contributes towards the development of novel diagnostic tools.

The following research questions were addressed.

i) **Molecular pathophysiology**. What is the composition of the secretome of a virulent *Babesia canis* strain on a genomic and proteomic level? Which are the molecular parasite components during acute virulent *B. canis* infection? Which secreted soluble and host membrane-bound proteins from blood-stage parasites are involved in parasite-host interactions?

ii) **Clinical pathophysiology**. Are there different measurable prognostic markers (host factors) associated with poor outcomes in acute *B. canis* infections?

iii) **Tick vector occurrence following an outbreak of *B. canis***. Is a pet-owner based tick sampling strategy applicable as a systematic and cost-effective sampling method to detect the tick vector for *B. canis* in an emerging epidemiological region? Which tick species are parasitizing companion animals?

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# PART III MANUSCRIPTS

## 1. General remarks

The thesis includes three first author manuscripts, of which two were printed in peer-reviewed journals and one is in preparation for being submitted. In addition, contribution toward novel diagnostic tools resulted in the generation of two monoclonal antibodies with diagnostic potential. All performed work would not have been possible without the fruitful collaboration and contributions by the co-authors and acknowledged persons. The data from this thesis contributed toward the evaluation of a patent announcement in collaboration with the technology transfer organization from the University of Zurich 'Unitectra' and industrial partners.

Chapter 2.1. is the first draft of the manuscript on the molecular characteristics presented during acute and virulent *B. canis* infection. This study represents the main part of my PhD work. Substantial proportion of this work including generation of biological material, evaluation and optimization of protocols, laboratorial and bioinformatics work as well as analysis and interpretation of the results was performed by me, in collaboration with Dr. Chandra Ramakrishnan (transcriptomics) and supervised by Prof. Adrian B. Hehl. Fundamental part for the success of this work consisted of the challenging animal experiments supervised intensely by Prof. Peter Deplazes. Genome assembly and annotation was assisted by Dr. Giancarlo Russo (Functional Genomics Center Zurich). The manuscript draft was mainly written by me and Prof. A.B. Hehl and will be improved by few additional data.

Chapter 2.2. is the published manuscript in 'Journal of Veterinary Internal Medicine' with the title "Prognostic markers in acute *Babesia canis* infections". This clinical orientated work evaluated prognostic markers associated with poor outcomes in acute infections and gives insights into the pathophysiology of canine babesiosis. Supervised by Prof. Peter Deplazes and assisted by the Clinical Laboratory from the Vetsuisse Faculty, University of Zurich (UZH), I examined and analyzed clinical cases and samples from the Clinic for Small Animal Internal Medicine (UZH), private practices and experimental inoculated dogs. I was substantially involved in the design of this work and performed all the included animal experiments. All authors contributed in drafting the manuscript.

Chapter 2.3. corresponds to the published manuscript in 'Ticks and Tick-borne Diseases' with the title "Ticks on dogs and cats: A pet owner-based survey in a rural town in northeastern Switzerland". This survey demonstrated a simple and cost-effective method for sampling vector-ticsk verified by standard tick-flagging techniques and identified a so far unknown focus of the vector-tick in an area with increased incidence of *B. canis* babesiosis. The project idea, designs, and strategies were developed by Prof. Peter Deplazes and me, and was further supervised by Prof. Alexander Mathis from the Institute of Parasitology (UZH). I performed the morphological and molecular tick identifications, carried out the field investigations, and conducted the statistical analyses. All authors contributed in drafting the manuscript.

# 2. Manuscripts

## 2.1. Combined analysis of the *Babesia canis* genome, transcriptome and secreted proteome during virulent infection identifies potential pathogenicity factors

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**Abstract**

Infections of dogs with virulent strains of *Babesia canis* are characterized by rapid onset and high mortality, comparable to complicated human malaria. As in other apicomplexan parasites, most *Babesia* virulence factors responsible for survival and pathogenicity are secreted to the host cell surface and beyond where they remodel and biochemically modify the infected cell and interact with host proteins in a very specific manner. Here, we investigated factors secreted by *B. canis* during acute infections in dogs and report on in silico predictions and experimental analysis of the parasite's exportome. As a backdrop, we generated a fully annotated *B. canis* reference genome sequence of a virulent Hungarian field isolate (strain BcH-CHIPZ) underpinned by extensive genome-wide RNA-seq analysis. We find evidence for conserved factors in apicomplexan hemoparasites involved in immune-evasion (e.g. VESA-protein family), proteins secreted across the iRBC membrane into the host bloodstream (e.g. SA- and Bc28 protein families), potential moonlighting proteins (e.g. profilin and histones), and uncharacterized antigens present during acute crisis in dogs. The combined data provides a first predicted and partially validated set of potential virulence factors exported during fatal infections, which can be exploited for urgently needed innovative intervention strategies facilitating diagnosis and management of acute and chronic canine babesiosis.

**Introduction**

Apicomplexan parasites have evolved a plethora of targeted interactions with their host organisms and cellular machinery to elicit modification of gene expression and cellular functions. These modifications are critical for invasion and optimizing intracellular niches for replication, differentiation, and persistence, while securing nutrient import and mediating immune evasion (Goldberg and Cowman 2010). As a result, host-parasite relationships in Apicomplexa can be viewed as co-adaptations driven by co-evolution of parasites with their hosts (Morrison 2009). Most importantly, this complex interplay is directly linked to pathogenicity and virulence which characterize symptomatic infections with these protozoan parasites (Bradley and Sibley 2007; Haldar and Mohandas 2007; Hutchings et al. 2007; Maier et al. 2008; Lobo et al. 2012; Singh et al. 2014).

Three genera of this phylum represent a triad of phylogenetically related hemoprotozoa: *Plasmodium* (human and animal malaria), *Theileria* (theileriosis in livestock), and *Babesia* (severe disease and death in cattle, horses, dogs, with some zoonotic potential) are the causative agents of important human and/or animal diseases with high morbidity and mortality, responsible for significant economic burdens and healthcare challenges worldwide (Lau 2009). The clinically relevant part of the complex *Babesia* life cycle that involves tick vectors in addition to mammalian hosts takes place during asexual proliferation of parasites in red blood cells (RBC). This represents a distinct specialization of *Babesia* for life in mammalian erythrocytes, i.e. cells which lack organelles, as well as endocytic and exocytic membrane transport machinery (Gohil et al. 2010; Spielmann and Gilberger 2015).

Canine babesiosis is an emerging tick-borne disease of dogs with global distribution caused by morphologically distinctive *Babesia* species (Schetters et al. 1997; Boozer and Macintire 2003; Irwin 2009). The distribution of disease caused by *B. canis*, the species responsible for a virulent form of canine babesiosis in Europe and Western Asia, appears to spread northward, as demonstrated by an increasing numbers of cases in areas outside of classical endemic regions (Halos et al. 2014). The clinical spectrum of symptomatic *B. canis* disease is broad, ranging from apparently silent and mild infections to fulminant and often fatal, human malaria-like presentation (Irwin 2009). Indeed, in many respects canine babesiosis can serve as model for clinical human malaria (Reyers et al. 1998; Schetters and Eling 1999). Reports from Central and Eastern Europe (e.g. Hungary) indicated a high incidence of canine babesiosis with severe symptoms and high mortality rates (12 – 20%), whilst uncomplicated forms with mortality rates below 5% prevail in Southern and Western European countries (Italy, Spain, Portugal, Netherlands, France) (Furlanello et al. 2005; Bourdoiseau 2006; Mathe et al. 2006; Ruiz de Gopegui et al. 2007; Solano-Gallego et al. 2008; René-Martellet et al. 2013). This suggested genetic heterogeneity within the *B. canis* population structure (Carcy et al. 2015) which was further supported by reduced to absent cross-immunity in vaccination trials with soluble parasite antigen (SPA) extracts prepared from isolates with different geographic origins (Schetters 2005). In addition, severe side

effects with SPA formulations suggested the presence of highly pathogenic factors in many of these uncharacterized crude fractions (Adaszek et al. 2012). However, the nature of these soluble virulence factors which are suspected to cause severe pathogenesis in natural infections of dogs remains unknown.

Analysis of correlated genomic, transcriptomic and proteomic data provides the molecular underpinnings for investigating the role of exported factors in disease manifestation and progression. However, experimental investigation of *B. canis* biology is severely limited by the current lack of cultivation techniques for parasite propagation *in vitro*. Here, we implemented short term *in vitro* cultivation of infected RBC in combination with genome sequencing, annotation, and comparative genomic characterization of a virulent Hungarian *B. canis* field isolate. By correlating RNA-seq and protein mass-spectrometry data, we provide the first account of factors exported by *B. canis* blood stages with potential links to host-pathogen interaction and acute virulence.

## Results

### *Genome sequence of a virulent* Babesia canis *strain*

Until recently, only few genes from canine *Babesia* species have been characterized and global genomic data were not available. To provide the molecular underpinnings for targeted approaches to diagnosis, vaccination and clinical management of canine babesiosis we undertook complete sequencing, assembly, annotation and characterization of the genome of a virulent *B. canis* strain, designated BcH-CHIPZ. The BcH-CHIPZ isolate originated from the blood of a Swiss dog that was infected, presumably through the bite of an infected tick, during a trip to Hungary. The isolate was characterized by genotyping and identified as double B-type by restriction site analysis in the Bc28-multigene family and the 18SrRNA gene (Carcy et al. 2015). We implemented a next-generation high throughput approach (PacBio® SMRT) for sequencing of BcH-CHIPZ genomic DNA prepared after enrichment of infected RBC (iRBC). Assembly of the parasite-specific sequence reads after elimination of all reads from host DNA and gap-filling yielded 43 high-quality scaffolds covering a total of 7'002'180 bases with a median weighted contig size (N50) of 185 kb. Hence, the calculated size of the *B. canis* genome lies between the phylogenetically related *B. microti* (6.5 Mb) and the *B. bovis* (8.2 Mb) genomes (Brayton et al. 2007; Cornillot et al. 2012). The main features of the annotated *B. canis* genome and a comparison with other apicomplexan genomes are presented in Table 1. The quality of the genome assembly based on the presence of core eukaryotic genes (Parra et al. 2007; Reid et al. 2014) indicates the *B. canis* genome sequence to be 92.6% complete. This is comparable with the genome of the *T. gondii* ME49 type II reference strain (Gajria et al. 2008) and consistent with values of other *Babesia* genome sequences, which lie between 91.7% and 95.6% (Supplemental_Document_S1).

This robust annotation of species-specific gene models was based on the analysis of orthologues and synteny in related species (described below). Furthermore, the *B. canis* scaffolds could be contiguously aligned to the 4 chromosomes of the related *B. bovis* by a whole genome mapping approach. This suggested a strong syntenic relationship with chromosomes of *B. bovis* (Fig. 1).

*Genome-wide comparison of* B. canis *gene models with those of other Apicomplexa reveals species-specific genes*

The *B. canis* gene models were analyzed for orthologues in four representative apicomplexan parasites *B. bovis*, *Th. annulata*, *P. falciparum*, and *T. gondii* and the fully sequenced *Babesia* species *B. bovis*, *B. bigemina*, and *B. microti* by clustering of orthologous groups using a Markov Cluster algorithm in OrthoMCL (Fig. 2). All four *Babesia* species share a core set of 1216 conserved orthologous clusters, even allowing for the smaller number of gene models in *B. canis*. However, comparison of bovine and rodent *Babesia* gene models revealed 1030 shared OrthoMCL clusters which are not represented in the *B. canis* genome, which comprise a higher than average proportion (75.7%) of hypothetical, putative, or uncharacterized genes (Supplemental Data S1, S2). We take this comparatively smaller and more diverged set of annotated genes in *B. canis* as a reflection of the narrow and highly specific host range in vertebrates and ticks. This interpretation is based on the premise that the diversity of encoded proteins is linked to the degree of specialization for different environments, niches, and lifestyles of a parasite, thus reflecting lineage-specific adaptations (Templeton et al. 2004; Heitlinger et al. 2014). Therefore, the divergent and less redundant (smaller) gene set may represent the comparatively narrow vertebrate and arthropod host range of *B. canis*. Interestingly, with 347 genes/Mb the *B. canis* genome has a similar gene density as the *B. bovis* (372 genes/Mb) and *B. microti* (354 genes/Mb) genomes, although some regions appear to be more densely packed than others (Fig. 1).

Analysis of orthologues revealed a high degree of synteny between the genomes of *B. canis* and *B. bovis* compared with the genome of rodent *B. microti* (Fig. 1).

*Analysis of mRNA expression and prediction of the* B. canis *exportome*

Exported proteins of apicomplexan parasites include virulence factors, i.e. parasite-specific proteins which interfere directly with host cell functions. As mature RBC lack de novo protein synthesis or trafficking pathways, membrane transport machinery for targeting these proteins via the cytoplasm to the iRBC surface and beyond are provided by the parasite. These parasite-induced cytoplasmic alterations of iRBC are critical for its development and directly linked to the severity of babesiosis (Pedroni et al. 2013). As a first step to characterize parasite-secreted factors and surface proteins, we generated a predicted *B. canis* exportome based on the annotated BcH-CHIPZ genome. We used data

mining tools to search for genes coding for predicted parasite surface and iRBC targeted factors. The criteria and prioritization for this selection were based on the presence of a canonical hydrophobic N-terminal signal peptide (cSP) sequence, an alternative secretion pathway (a/ncSP) signal used by non-classical protein secretion, predicted transmembrane (TM) domain(s), a GPI anchor signal, or previously described domains of conserved apicomplexan secretory proteins and protein families (for details see Materials and Methods section; Supplemental Fig. 1; Supplemental Data S3). By this approach we generated a curated and parsed list of 509 predicted proteins exported to the parasite surface and/or to the host, which corresponds to 14.6% of all gene models (Fig. 3A). This proportion is very similar to the 528 gene models (14%) representing the predicted *B. bovis* secretome (Pelle et al. 2015).

All apicomplexan parasites express members of gene families that are exported to the surface of invasive stages or the plasma membrane of iRBC where they interact with the host immune system (Spence et al. 2013). Members of the *Babesia* variant erythrocyte surface antigen (VESA) family on the surface of iRBC (Allred et al. 1993) comprise the largest protein family in *B. canis* (103 gene models) in the predicted exportome (Fig. 3B). Most VESA genes cluster together on few contigs (Fig. 1), unlike other gene families of the predicted exportome which are distributed more randomly. Furthermore, VESA genes are in regions with high gene density, consistent with frequent gene duplication events by a driving selection pressure on these proteins associated with immune-evasion. MSA, *B. canis* merozoite surface-exposed, plasma membrane-anchored proteins (2 gene models in *B. canis*), as well as Bc28 family members (20 gene models), are highly abundant on the merozoite surface but are also shed and interact with the iRBC membrane and the host's immune system (Yang et al. 2012; Zhou et al. 2016). The second-largest family of exported proteins comprises secreted DnaJ (Hsp40) chaperones (26 members). *P. falciparum* DnaJ proteins have a demonstrated role in remodeling of iRBC and in pathogenesis among other functions (Rug and Maier 2011; Njunge et al. 2013). The so-called "secreted antigens" (SA-1 and SA-3, 24 members) comprise yet another family of genes coding for exported proteins. PBlast analysis showed that the *B. canis* SAs are highly homologous (23 of 25 proteins with e-values <10E-5) to those of *B. gibsoni,* which also propagates in dogs, and to a lesser extent to SAs of *B. bigemina* (3 of 25 proteins with e-values <10E-5), which infects bovines, suggesting that host-specific factors drive expansion and diversification of these orthologous gene families. In contrast, genes coding for smORF and spherical body proteins (SBP) appear to be specific to *B. bovis* and are without orthologues in the *B. canis* genome (Jackson et al. 2014; Pelle et al. 2015). Proteins of the "secreted antigen family", as the name indicates, are secreted beyond the iRBC and circulate in the blood of infected dogs but are otherwise not well characterized (Zhou et al. 2016). The predicted *B. canis* exportome also includes a group of 23 protein kinases. Of note, among the group of secreted protein kinases and pseudokinases are key virulence factors in toxoplasmosis (Saeij et al. 2007; Steinfeldt et

al. 2010; Fleckenstein et al. 2012; Etheridge et al. 2014). Kinases are also secreted into iRBCs in *Plasmodium*, where they are responsible inter alia for remodeling the host cell (Saeij et al. 2006; Lim et al. 2012). However, functional data related to secreted kinases in *Babesia* are not available. Nevertheless, kinase inhibitors have been identified as potential novel drugs for the treatment of babesiosis in cattle (Bork et al. 2006; Pedroni et al. 2016).

To refine the predicted host-targeted *B. canis* exportome dataset we analyzed the transcript levels of all selected 509 gene models in parasites harvested from a splenectomized dog. Evidence from a genome-wide RNAseq analysis showed transcription of 71.5% of the 509 exportome gene models (FPKM > 10; n=364) (Fig. 4A). Remarkably, only a few members each of the five most abundant exported protein family genes (VESA-, DnaJ-, SA-, PK-, and Bc28- multigene families) with potential roles in antigenic variation and immune evasion were expressed at high levels (FPKM > 1000) (Fig. 4B-F). The particular expression profile of the five gene families in this snapshot might be due to selection of a few specific phenotypes in the population at the peak of pathogenicity, but one should also consider that the collected blood stage parasites do not represent a synchronously developing population. Nevertheless, this finding merits further investigation, in particular in the light of previous observations indicating that pathogenicity was associated with expression of a specific subset of VESA- and other exported genes in *B. bovis* infection (Pedroni et al. 2013).

Further in silico characterization of the predicted B. canis exportome revealed a significant number of proteins (210/509) lacking a canonical hydrophobic signal sequence for co-translational insertion into the endoplasmic reticulum lumen, which is suggestive of non-classical secretion. This export pathway is generally known as 'leaderless protein secretion' (Nickel 2003; Bendtsen et al. 2004) and was described recently also in *P. falciparum* (Lingelbach and Przyborski 2006; Sudarsan et al. 2015; Thavayogarajah et al. 2015). We screened the *B. canis* exportome for additional motifs associated with protein trafficking into the host cell and beyond. We searched specifically for matches to *Plasmodium* host targeting (HT) motifs or PEXEL export elements (RxLxE/Q/D) for trafficking beyond the parasitophorous vacuolar membrane (PVM) (Hiller et al. 2004; Marti et al. 2004), which have been also identified in *T. gondii* and *C. parvum* (Hsiao et al. 2013; Pelle et al. 2015). Although *Babesia* builds a PVM during invasion of RBC it disappears within minutes and the parasite resides directly in the host cell cytoplasm (Asada et al. 2012). A comprehensive comparative in silico analysis of protein export across 10 Apicomplexa species revealed PEXEL-like motifs (PLM) in *B. bovis* (but not in *T. parva)* with a role in retention of these proteins in so called spherical bodies and release in a cell-cycle dependent manner (Pelle et al. 2015). We found hits for the *B. bovis* PEXEL-like motifs within the first 100 N-terminal amino acids in 64.3% of cSP-containing proteins and only in 7.6% of predicted alternatively secreted *B. canis* proteins (Supplemental Data S3). Using a hidden Markov model to identify alternative motifs by de novo pattern discovery did not yield any additional hits.

*Shotgun proteomics and transcriptomics identify potential* B. canis*-specific secreted virulence factors during severe disease*

Proteins involved in parasite-host interactions at acute crisis were identified by a comparative shotgun approach from *B. canis* BcH-CHIPZ infected and healthy host blood samples. Mass spectrometry datasets were generated from secreted soluble factors collected from pooled short-term cultures from three experimentally infected dogs at acute crisis and RBC cultures derived from healthy blood donors, as well as from RBC membrane fractions from experimentally infected animals and non-infected controls. Using stringent thresholds to include only MS spectra with high peptide counts and after filtering the data with the non-infected control sets we identified a set of abundant parasite-specific proteins in these fractions. Based on their abundance in the iRBC and/or culture supernatant the identified secreted membrane-bound and soluble parasite proteins were considered candidate factors involved in host-pathogen interaction (Table 2; Supplemental Fig. S2).

Not surprisingly, considering the limited understanding of apicomplexan pathogenicity determinants, the resulting *B. canis* dataset of abundant exported proteins comprises 47.8% (11/23) hypothetical proteins. The annotated proteins were parsed as follows: Factors on cell surface membranes (from the parasite or the iRBC) which include 2 variant erythrocyte surface antigens (VESA) located on the surface of iRBC, a *Babesia* spp. membrane protein (homologous to *B. bovis*, *B. microti*, *B. bigemina*, *Theileria annulata*, *T. equi*, *T. orientalis*, *T. parva*, and *Cytauxzoon felis*), a spectrin repeat superfamily extracellular matrix binding protein with homology to *B. bovis* VESA, and 2 merozoite surface antigens of the Bc28 gene family. These proteins where shown to be involved in survival and virulence of several hemoprotozoa species (Foley et al. 1994; Brayton et al. 2007; Gohil et al. 2010; Lau et al. 2011; Yang et al. 2012; Carcy et al. 2015). The dataset comprises SA1 and SA3, belonging to the above described third-largest exported family of proteins (secreted antigen family) suggesting a role in host-parasite interaction. The *B. canis* dataset also contains profilin, a secreted virulence factor described in *P. falciparum* and *T. gondii* with important roles in the process of crossing biological barriers during host cell invasion and egress (Kursula et al. 2008; Plattner et al. 2008), a SET-domain containing protein with putative function in lateral gene transfer in apicomplexans (Kishore et al. 2013), and a SNARE protein mediating membrane fusion of secretory vesicles with the plasma membrane (Ayong et al. 2007; Parish and Rayner 2009). Furthermore, a secreted histone protein (classified as DNA-binding protein) was detected. Histone proteins were described to have moonlighting functions outside of the nucleus in *P. falciparum*, specifically at the interface between parasite and erythrocyte cytoplasm, i.e. the parasitophorous membrane (Luah et al. 2010).

All hits in the proteomic dataset for which GO descriptions or blast homologies could be assigned were further validated using transcriptome evidence (threshold FPKM value > 10). Only Bc28.2, a known secreted *B. canis*-specific protein (Yang et al. 2012), fell below this threshold with a measured FPKM

of (4.5). However, the correlation between transcript- and protein abundance is not linear and there is a complex relationship leading to notable discrepancies due to a range of factors such as protein- and mRNA turnover (Wastling et al. 2012). Taken together with additional evidence from the literature, these proteins are good candidates for factors that are presented to the host on the parasite or iRBC surface, or released in to the bloodstream and potentially involved in parasite persistence and virulence.

*Correlating parasite gene expression and disease parameters of* B. canis *babesiosis at acute crisis*

We collected biological material from three experimentally infected dogs, which all showed very similar rapid disease progression consistent with fatal babesiosis (described in Eichenberger et al. 2016). The first mild clinical signs (e.g. lethargy) preceded acute crisis with early symptoms of septic shock by a maximum of 27 hours (Supplemental Fig. S3). In spite of the fulminant disease manifestation, a hallmark of canine babesiosis is a remarkably low-grade parasitemia (Schetters et al. 1997; Furlanello et al. 2005; Schetters et al. 2009) typically with <2% infected erythrocytes observed in the peripheral blood. The erythrocytic stages of the parasite are entirely responsible for the morbidity and mortality associated with canine babesiosis. Morphological characterization of blood stage parasites revealed pleiomorphic forms in Giemsa-stained blood smears derived from a critically ill dog (Fig. 4A). Small and large ring stages were the most frequently detected forms, while only few classically pear-shaped forms could be identified. To characterize the parasite stages in iRBC from experimentally infected animals on a molecular level in more detail, we used the high-throughput RNA-seq data to identify orthologous gene models with stage-specific expression in *Plasmodium* spp. We intersected the list of *B. canis* gene models with evidence for expression with a list of validated *Plasmodium spp.* stage-specifically expressed genes (e-value threshold of 10E-3) (Supplemental Data S4). We found evidence for RNA-seq reads (threshold FPKM>10) mapping to 22 of 29 conserved merozoite-, and 4 of 7 early gametocyte genes in a blood sample collected from a splenectomized dog (Fig. 4B). The higher abundance of merozoite- (proliferative) and early gametocyte-specific orthologues (sexual stage) during severe disease was consistent with the microscopy data and suggested a paucity of circulating sexual stages in this sample. Nevertheless, some evidence for early gametocyte stages was found, which could indicate that acute clinical crisis in infected dogs might trigger development of sexual stages. For example in *Plasmodium*, gametocyte formation occurs in bone marrow and aggravates clinical symptoms and disease outcome (Aguilar et al. 2014; Joice et al. 2014). A potential bone marrow tropism could explain typical clinical signs, e.g. leucopenia and thrombocytopenia, associated with poor outcome in acute *B. canis* infections (Eichenberger et al. 2016). *B. canis* orthologues of the 18 identified invertebrate stage-specific proteins (flagellar “late” gametocyte-, ookinete- and sporozoite genes) described for *Plasmodium* had FPKM values between 0

and 10.8, indicating that no or only insignificant amounts of these mRNAs were detected in the sampled parasite population. Based on the degree of orthologue conservation and the microscopy data we interpret this as paucity of late gametocyte stages in the peripheral blood (Supplemental Data S4).

Comparative analysis of the *B. canis* secreted- and RBC-associated proteomes revealed members of the Bc28 and SA protein family. The annotation for the *B. canis* BcH-CHIPZ genome contains 24 gene models coding for members of the SA- protein family. By aligning the SA-family members using Clustal Ω, shared amino acid motifs at the N-terminal part of the predicted proteins were revealed (Supplemental Fig. S4). Some SA genes have been identified in other *Babesia* species (Jia et al. 2006; Jia et al. 2009; Luo et al. 2011; Sevinc et al. 2015). However, this gene family appears to be expanded in *Babesia* species infecting canines: cluster analysis with *B. bovis*, *B. bigemina*, and rodent *B. microti* genome data did not identify shared clusters, whereas pBLAST searches showed high homology to two known *B. gibsoni* secreted antigens (SA-1 and SA-3; Supplemental Data S5).

Currently, two members of the Bc28 gene family are characterized as major merozoite surface antigens playing a critical function in the interaction of merozoites with RBC (Carcy et al. 2006; Yang et al. 2012). The 20 *B. canis* family members (including the two characterized Bc28 proteins) which clustered in OrthoMCL were aligned using Clustal Ω (Supplemental Fig. S4 B). Our data support the previous classification of Bc28.1 (Yang et al. 2012) as a GPI anchored membrane protein. Other Bc28.1 family members lack both GPI-anchor signal and transmembrane domain, but are still are predicted to be exported by virtue of a canonical hydrophobic signal peptide or an alternative route (Fig. 3).

**Discussion**

Secreted soluble and membrane-targeted parasite proteins have important roles at the host-parasite interface and are frequently virulence and pathogenesis factors. Systematic investigation of these interactions in infections with potentially fatal blood-borne parasites, such as the difficult to cultivate *Babesia canis*, which modify the host cell, its microenvironment, as well as host physiology in multiple ways, is a challenge. Moreover, these questions cannot be addressed with biological material from regular patients from an animal clinic. Hence, in this study, we used experimental infections of dogs in a controlled setting to gather data on disease progression, clinical parameters, and parasite development. We report first genome and gene expression data of the apicomplexan *B. canis* with a focus on exported factors secreted during acute infection. Acute *B. canis* infections with fulminant disease progression is associated with high mortality and characterized by an excessive inflammatory response caused by protozoal sepsis. This is caused by dysregulation of pro- and anti-inflammatory mechanisms during the acute phase of the disease, resulting in shock and end-organ failure (Udomsangpetch et al. 1997; Clark et al. 2006; Matijatko et al. 2012). Interestingly, fatal *B. canis* infections (natural and experimentally induced) are invariably associated with at most moderate non-

hemolytic anemia and very low parasitemia (Schetters et al. 1997; Furlanello et al. 2005; Eichenberger et al. 2016). This strongly suggests that potent secreted parasite factors are responsible for the severe pathogenicity of canine babesiosis. Hence, these as yet uncharacterized, secreted virulence factors are prime targets for inclusion into vaccines but also in the development of diagnostic kits. A particular challenge in treating canine babesiosis is the combination of unspecific symptoms, late presentation, and rapid progression of the disease to a life-threatening stage. Thus, tools for early and accurate diagnosis of canine babesiosis as a prerequisite for initiating specific treatment and prevent fatal outcome are missing and are therefore a prime goal in clinical research. Secreted soluble antigens in acute *B. canis* infections circulate in the bloodstream from the onset of parasitemia and before specific antibodies are present, as seroconversion usually takes at least 8 to 10 days (Sibinovic et al. 1967; Bobade et al. 1989; Vercammen et al. 1995; Schetters et al. 1996). Given the generally very low parasitemia and difficult to identify pleiomorphic stages, these circulating *Babesia* antigens are prime targets for early and rapid diagnosis in acute infections. This principle was implemented recently in a species-specific rapid diagnostic test for acute malaria by detection of several secreted soluble histidine rich parasite proteins (HRP2) with monoclonal antibodies (Merwyn et al. 2011).

As a prerequisite for the study of parasite factors interacting with the host we report an annotated *B. canis* reference genome. Based on a next-generation high throughput approach (PacBio SMRT-sequencing) we generated 43 high-quality contigs at 55-fold average coverage with a total length of approximately 7 Mb. The quality of the genome annotation is key for subsequent gene expression analysis by RNA-seq or MS analysis of peptides. Annotation of *Bc*H-CHIPZ genome was based on a combination of *ab initio* gene predictions trained on high-quality annotated *P. falciparum*, *B. bovis*, and *Theileria annulata* genomes, and combined with *de novo* transcriptome data (assembled Illumina RNA-seq reads) from *B. canis* blood stages resulting in 3467 gene models. By mapping gene-clusters shared among hemozoa species we were able to assign all *B. canis* contigs to 4 *B. bovis* chromosomes. This suggests that the nuclear *B. canis* genome is likely organised on 4 chromosomes, similar to the genomes of the phylogenetically related *B. bovis* (Brayton et al. 2007), *B. bigemina* (Jackson et al. 2014), and *B. microti* (Cornillot et al. 2013). We found that 92.6% of the *B. canis* gene models are designated as core eukaryotic genes, which also map to the *Toxoplasma gondii* genome dataset (Parra et al. 2009), and 94.8% (1722/1822) of orthologous clusters can be identified in one or more annotated *Babesia* species. Taken together, we considered the *Bc*H-CHIPZ genome assembly and annotation as sufficiently robust for transcriptomic and proteomic analyses of secreted effectors. Considering that, similar to human malaria (Miller et al. 1994), the clinical presentation of canine babesiosis is caused by blood stage parasites alone, understanding the clinical manifestations of the disease requires identification and characterization of parasite antigens that interact with the host beyond the iRBC. To address this question we generated a high-quality blood-stage transcriptomic dataset (118 million

mapped reads on the *B. canis* genome) and a proteomic dataset representing a secreted- and membrane-associated protein fraction from a blood sample collected at the onset of acute crisis in experimentally infected dogs.

Several major secreted factors for acute pathogenicity and persistence of hemoprotozoan parasites, have been described in *P. falciparum* and *B. bovis* (Allred and Al-Khedery 2004). Following RBC invasion, *B. bovis* exports VESA proteins to the surface of the host cell modifying the iRBC membrane to induce cytoadherence of iRBC at the capillary and postcapillary venous endothelium (Allred et al. 1994; O'Connor et al. 1997; Allred et al. 2000). VESA expression is subject to rapid antigenic variation defined as the serial clonal replacement of the expressed VESA gene in daughter merozoites before invasion of a new RBC. This gives rise to antigenically distinct parasite populations, which complicates mounting of an effective host immunity. Antigenic variation, i.e. sequential exposure of different sets of surface antigens is likely a universal immune-evasion strategy among hematozoa and has been documented in several *Babesia* and *Plasmodium* species including *B. rodhaini*, *B. microti*, *B. bigemina*, *P. falciparum* and *P. vivax* (Allred and Al-Khedery 2006; Singh et al. 2014). Not surprisingly, the largest exported protein family in *B. canis* consists of the variant erythrocyte surface antigen (VESA) proteins. Indeed, a recent study using comparative transcriptomics and proteomics of attenuated and virulent *B. bovis* strains showed a significantly increased diversity of upregulated VESA genes in virulent strains (Pedroni et al. 2013). In the present study, we detected a high number (74/103) of transcribed VESA genes as well as their products in the *B. canis* secreted proteome. Hence, adherence of *B. canis* iRBC to endothelial cells is one explanation for the apparent discrepancy between the low parasitemia observed in peripheral blood samples and the severity of the disease, consistent with sequestration of parasites and removal from the peripheral circulation. Correspondingly, using parasites isolated from a peripheral blood sample for the transcriptomic and proteomic studies might introduce a bias with respect to the detection of important pathogenicity factors. Interestingly, only 6 of the 103 VESA gene models in *B. canis* are predicted to encode a classical N-terminal signal peptide, in line with data from *B. bovis* (7 out of 119) (Brayton et al. 2007), suggesting an alternative route for export of most of the members of this protein family.

Shedding of extracellular (exosome-like) vesicles (EVs) as membrane-bounded carriers for proteins, lipids and RNA for intra- and inter-species communication (reviewed in (Raposo and Stoorvogel 2013; Yanez-Mo et al. 2015) was identified recently as a non-classical export pathway for a completely new class of important parasite-derived factors. In *P. falciparum* infections the concentration of EVs in the blood is positively correlated with severe disease suggesting an important role in pathogenesis and immune modulation (Nantakomol et al. 2011). Strikingly, the *B. canis* BcH-CHIPZ genome encodes homologues of important structural proteins and enzymes frequently found in exosomes, including heat shock proteins (e.g. HSP70 and 90), tetraspanins and ALIX protein, TSG101/ESCRT protein, 14-3-

3 protein, thioredoxin peroxidase, histones, and the RAB GTPases 5, 7, 11, as well as a RAB GDI (Thery et al. 2002; Silverman and Reiner 2011; Raposo and Stoorvogel 2013). Based on their biogenesis, exosomes often contain endosome-associated proteins, such as a *B. canis* SNARE protein detected in the culture supernatant. In *P. falciparum* a SNARE protein was localized proximal to the plasma membrane where it is possibly involved in membrane trafficking events associated with the parasite's food vacuole (Parish and Rayner 2009). Hence, EVs in *B. canis* infections could be crucial vehicle carriers for the dissemination of pathogenicity factors that are linked to acute and severe disease, including coding and non-coding RNA which can interfere with host cell gene expression. In fact, secretion via EVs could account for many of the non-classically secreted proteins detected in the *B. canis* proteomic datasets. We identified several proteins with moonlighting functions which have been identified as virulence factors in related Apicomplexa. For example profilin (pfn) was shown to have important roles in the process of crossing biological barriers during host cell invasion and egress, in addition to its classical role as a regulator of actin dynamics (Plattner et al. 2008). The structure of the *P. falciparum* pfn accommodates additional domains with as yet unknown functions, which are potentially involved in the parasite-host interplay (Kursula et al. 2008). Another interesting finding was a histone protein in the iRBC membrane fraction. Histones are classically localized in the cell nucleus with functions in chromatin remodeling and transcriptional regulation. In several *Plasmodium* species, a histone protein methylated at position L9 was detected in the PV membrane (Luah et al. 2010). The conserved PVM localization in *Plasmodium* indicated that histones have evolved additional functions as virulence factor in invasion and/or interaction with the host. As *Babesia* are not contained within a PV, the potential moonlighting function of this protein will have to be further investigated. Furthermore, histones are commonly found in large EVs (Colombo et al. 2014). We also detected a *B. canis* SET-domain containing protein (BcSET) in the secreted soluble fraction. SET proteins are involved in diverse mechanisms such as transcriptional regulation, enhancer function, mRNA splicing, DNA replication, and DNA damage response (Herz et al. 2013). An exported SET exerting an immune-suppressive function by transcriptional silencing of host gene expression was described in the pathogen *Bacillus anthraxis* (Mujtaba et al. 2013). Interestingly, deregulation of gene expression due to SET domain-containing proteins is correlated with unfavorable clinical outcomes in various forms of cancer (Greer and Shi 2012).

Members of the Bc28 and SA gene families are among the most prominent *B. canis*-specific secreted factors, and have recently gained attention as candidates for innovative vaccine strategies and in diagnostics (Yang et al. 2012; Zhou et al. 2016). Bc28 gene products are highly represented on the merozoite surface, but are also shed and can interact with the iRBC membrane (Yang et al. 2012). Bc28 belong to a multigene family composed of polymorphic genes. Different *B. canis* strains in Europe are associated with different mortality rates and show genetic heterogeneity in Bc28.1, detectable by a

PCR-RFLP test (Carcy et al. 2015). It has been suggested that this variable repertoire has evolved to allow the parasite to evade host immune responses, although merozoite surface proteins of *Babesia* spp. are thought to be the main targets of the adaptive host immune response (Carcy et al. 2006; Goo et al. 2012). However, whether all members are directly involved in immune evasion and virulence, or even show stage-specific behavior, will have to be investigated in more detail.

In the absence of reverse genetic techniques for *B. canis* research the sequencing and annotation of a virulent *B. canis* strain together with the described parasite blood stage proteome and transcriptome provides a powerful underpinning for designing targeted intervention strategies such as the development of novel diagnostic tools, effective vaccines, and innovative drugs.

## Methods

*Parasites*

Biological material for the different experiments were collected from experimentally infected dogs. Animals were inoculated intravenously with 1 x $10^6$ parasitized erythrocytes from a cryo-conserved field isolate, derived from a naturally infected Bernese mountain dog from Switzerland that had been exposed during a trip to Hungary. All experimentation with animals was in complete compliance with the strict Swiss animal welfare laws and regulations (Permit 122/2012), issued by the Veterinary Office of the Canton of Zurich, and round the clock surveillance by a veterinarian. Whole blood was treated with citrate-phosphate-dextrose-adenine (12.3% CPDA-1, v/v) for anticoagulation under sterile conditions and prepared immediately after euthanasia. Blood samples for genomic and transcriptomic studies were collected from an experimentally inoculated splenectomized dog (4 years old). Splenectomy increased the final amount of parasites in the peripheral blood to approximately 4% infected erythrocytes. The biological material for the proteome analysis was collected from three experimentally infected adult beagles with a parasitemia of 1 – 1.75% (Supplemental Fig. S2).

*Genome sequencing, assembly, and annotation pipeline*

Genomic DNA (gDNA)was prepared from purified and host leukocyte-depleted (Sriprawat et al. 2009) infected blood from an experimentally infected, splenectomized dog. High-throughput genome sequencing was performed on a PaciBio Single Molecule Real Time (SMRT) sequencing platform (Eid et al. 2009) run on 4 SMRT cells. 5 µg of input gDNA was used for library construction. Sequenced fragments were filtered to eliminate *Canis familiaris* reads and processed for assembly with PacBio read-alignment software BLASR (Chaisson and Tesler 2012). The final draft of the genome was obtained using a two-step procedure. De novo assembly was initially performed using the HGAP2 algorithm (Chin et al. 2013) and the outcome was further polished using the PacBio proprietary analysis package Quiver, a reference-guided consensus tool used as part of the SMRT analysis. The resulting

draft was subjected to gap-closing and further scaffolding using PBJelly v14.1.15 (English et al. 2012). Genome annotation was performed using different sources of evidence-based prediction on the Maker2 pipeline (Stanke and Waack 2003), on ab initio gene predictor algorithms (Korf 2004) with *P. falciparum* 3D7, *B. bovis* T2Bo, and *T. annulata* Ankara gene models as templates, and on Trinity-assembled de novo transcriptome evidence (Haas et al. 2013). Repetitive genomic elements were identified and masked from annotation with RepeatMasker using the full Repbase database (Jurka et al. 2005). Redundant transcripts potentially representing sequencing errors or genetic polymorphisms were clustered with the cd-hit software (Fu et al. 2012). Non-overlapping sequences of the different evidence datasets were generated from the gff-files with BEDTools genome arithmetic software (Quinlan 2014). Functional annotation of the gene-predictions was achieved by gene ontology mapping through Blast2Go v3.1 (Conesa et al. 2005) using a upper cut-off e-value of < 0.0005.
Full methodological details are provided in the Supplemental Material.

*Wet lab transcriptomics, and RNA seq data analysis*

Total RNA was prepared from host leukocyte-depleted *B. canis* blood stages using the Zymo Direct-zol RNA MiniPrep kit (Zymo Research) including an on-column DNase I (QIAGEN) digest. Paired-end, stranded sequencing of the cDNA library was performed on 1 lane of a Illumina HiSeq 2000 sequencer (Illumina Inc.). Measurement of expression level was based on fragments per kilobase of exon model per million mapped reads (FPKM), normalized by the length of the gene. A gene was considered expressed if its FPKM was at least 10. Full methodological details are provided in the Supplemental Material.

*Summary of computational genome analysis*

The identification of potential homologues of *B. canis* genes to selected apicomplexan parasites (*B. bovis* T2Bo, *B. bigemina* BOND, *B. microti* RI, *T. annulata* Ankara, *P. falciparum* 3D7, and *T. gondii* ME49) was carried out by protein blast (Blast+ executables v2.2.31) of each individual protein sequence. A e-value < 0.001 cut-off was considered appropriate. For comparative genomics analysis of species-specific genes and clustering of orthologues, OrthoMCL (v2.0.9) was used (Li et al. 2003; Fischer et al. 2011). Synteny analysis was performed in MCScanX (Wang et al. 2012) by the pairwise determination of syntenic regions between species based on the order of orthologues from the OrthoMLC output with default values. *B. canis* contigs were arranged according to the syntenic relationship to *B. bovis* with 'orderchr' execution in Circos utilities. Figure was drawn with Circos (Krzywinski et al. 2009). Prediction of the *B. canis* exportome was based on several in-silico analysis tools, databases, and manual curation (Supplemental Fig. S1). Full methodological details are provided in the Supplemental Material.

*Proteomic studies*

Proteomic analyses were based on secreted soluble *Babesia* antigens and membrane-bound *B. canis* proteins. Culture supernatant was collected after 18 h of short term in-vitro parasite cultures of *B. canis* RBC in serum-free medium prepared from anticoagulated whole blood from experimentally inoculated dogs. Similarly, blood from a healthy canine blood donor was cultured and supernatant was processed as a control. Isolation of membrane bound proteins was based on anticoagulated, leucocyte-depleted blood. IRBC were concentrated by Percoll (GE Healthcare) gradient centrifugation (Figueroa et al. 1990) and parasites were released from the iRBC by streptolysin-O mediated lysis (Richier et al. 2006) and removed by an additional Percoll gradient centrifugation step (Rodriguez et al. 1986). Membrane proteins were collected by homogenization and repeated ultracentrifugation steps, mild sonication and acetone precipitation. The same protocol was used to collect proteins from uninfected erythrocyte membranes. All samples were collected and stored at -80°C until further use.

Protein preparations were separated on one dimensional SDS-PAGE under sterile and reducing conditions. Each stained gel was cut into 8 equal sections and in-gel trypsin digested for subsequent mass spectrometry analysis. Liquid chromatography-MS/MS was performed on a Q-exactive mass spectrometer (Thermo Scientific) equipped with a nanoAcquity UPLC (Waters Corporation).

Following peptide data acquisition, searches were performed using the MASCOT search program against a database build on the *B. canis* annotated genes with a concatenated decoy database supplemented with commonly observed contaminants and the Swissprot database to increase database size, or against NCBI database for *Canis lupus familiaris*. Identified hits were filtered in Scaffold Viewer V4 (Proteome Software, Portland, US) based on stringent parameters to reach false discovery rates of <5%. Furthermore, all datasets were corrected for obvious host- and environmental contaminants by an additional pBLAST search of the NCBI non-redundant protein sequences database with the single peptide hits.

**Data access**

Sequences and data were deposited for public access on the ProteomeXchange platform via PRIDE (http://www.proteomexchange.org). The annotated *B. canis* genome is being made accessible through PiroplasmaDB (http://piroplasmadb.org/piro/).

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**Disclosure declaration**

Experiments with animals were conducted according to Swiss animal rights and regulations standards and approved by the Cantonal Veterinary Office of Zurich (permission number 122/2012) prior to the study. The authors disclose no conflict of interests.

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## Tables

**Table 1.** Genome characteristics of related apicomplexan hemoprotozoa

| Features | Species | | | | |
|---|---|---|---|---|---|
| | ***B. canis*** **[CHIPZ]** | ***B. bovis*** **[T2Bo]** | ***B. microti*** **[RI]** | ***Th. annulata*** **[Ankara]** | ***P. falciparum*** **[3D7]** |
| Genome size (Mb) | 7 | 8.2 | 6.5 | 8.4 | 23.3 |
| # of chromosomes | 4 | 4 | 4 | 4 | 14 |
| # of scaffolds | 43 | | | | |
| G+C content (%) | 45.8 | 41.5 | 36 | 32.5 | 19.4 |
| Protein coding genes | 3467 | 3706 | 3513 | 4082 | 5383 |
| Mean gene length (bp)[1] | 1044 | 1503 | 1327 | 1602 | 2292 |
| % of coding regions | 51.7 | 68 | 73 | 73 | 53 |
| Gene density[2] | 2020 | 2194 | 1816 | 2199 | 4374 |

[1]excluding introns and UTRs

[2]Genome size/number of protein coding genes

**Table 2**. List of *B. canis* specific candidates from culture supernatant derived and membrane-bound protein fractions.

| Identification | GO-description[1] | SP | TM | GPI | AS | Apicomplexan orthologue[2] | Transcriptome evidence (FPKM) |
|---|---|---|---|---|---|---|---|
| *Specific* B. canis *secreted soluble proteins:* | | | | | | | |
| BcH000240 | hypothetical protein, conserved | 1 | 6 | 0 | 0 | | no (0.7) |
| BcH000493 | N/A [VESA] | 0 | 0 | 0 | 1 | VESA (*B. bovis* BBOVII004140) | yes (673.5) |
| BcH001113 | hypothetical protein, conserved | 0 | 0 | 0 | 0 | | yes (544) |
| BcH002928 | hypothetical protein, conserved | 1 | 0 | 0 | 0 | | yes (64.2) |
| BcH003445 | Secreted antigen 3 | 0 | 0 | 0 | 1 | Secreted antigen 3 (*B. gibsoni* BAH70325) | yes (26.4) |
| BcH000300 | Merozoite surface protein (Bc 28.2) | 1 | 0 | 0 | 0 | *B. canis* specific | no (4.5) |
| BcH002050 | SET domain-containing protein | 0 | 0 | 0 | 0 | SET- and MYND domain containing protein, putative (*B. bigemina* BBBOND0302680) | yes (147.6) |
| BcH002986 | hypothetical protein | 0 | 0 | 0 | 0 | | yes (60.1) |
| BcH003048 | Profilin | 0 | 0 | 0 | 0 | Polypeptide of profilin (*B. bigemina* BBBOND_0104160) | yes (100.6) |
| BcH002141 | Snare protein | 0 | 1 | 0 | 0 | Snare protein, putative (*B. bigemina* BBOND0107960) | yes (236.6) |
| BcH000974 | hypothetical protein, conserved | 1 | 0 | 0 | 0 | VESA (*B. bovis* BBOV_III006920) | no (4.1) |
| BcH000821 | spectrin repeat superfamily | 0 | 1 | 0 | 0 | VESA (*B. bovis* BBOV_II004140) | yes (123.2) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| BcH000175 | extracellular matrix binding hypothetical protein, conserved | 0 | 0 | 0 | 0 | | yes (57.9) |
| BcH002958 | N/A [membrane protein] | 0 | 0 | 0 | 1 | membrane protein, putative (*B. bigemina* BBBOND_0105580) | yes (56.4) |
| *Specific* B. canis *host-RBC membrane presented proteins:* | | | | | | | |
| BcH000821 | Spectrin repeat superfamily extracellular matrix binding | 0 | 1 | 0 | 0 | VESA (*B. bovis* BBOV_II004140) | yes (123.2) |
| BcH000300 | Merozoite surface protein (Bc 28.2) | 1 | 0 | 0 | 0 | *B. canis* specific | no (4.5) |
| BcH000299 | Merozoite surface protein (Bc 28.1) | 1 | 1 | 1 | 0 | *B. canis* specific | yes (18.3) |
| BcH002699 | Histone h2a | 0 | 0 | 0 | 0 | 41-2-protein antigen precurson, putative (*B. bigemina* BBBOND_0103700) | yes (31.9) |
| BcH001932 | hypothetical protein, conserved | 0 | 0 | 0 | 0 | | (yes 12.5) |
| BcH002268 | hypothetical protein, conserved | 0 | 0 | 0 | 0 | | yes (111.9) |
| BcH001655 | VESA | 0 | 0 | 0 | 1 | VESA (*B. bovis* BBOV_IV002850) | yes (884.1) |
| BcH001448 | hypothetical protein, conserved | 0 | 0 | 0 | 0 | | no (0.9) |
| BcH003376 | Secreted antigen 1 | 0 | 1 | 1 | 1 | Secreted antigen 1 (*B. gibsoni* ALE14558) | yes (142.5) |
| BcH000391 | hypothetical protein, conserved | 1 | 0 | 0 | 0 | | yes (331.2) |
| BcH000895 | hypothetical protein, conserved | 0 | 0 | 0 | 0 | | yes (26.4) |

GO: gene ontology; SP: signal peptide; TM: transmembrane domain; GPI: AS: alternative secretion: GPI: glycophosphatidylinositol: VESA: variant erythrocyte surface antigen

[1] automated annotation by Blast2GO; [if no Blast2GO annotation could be assigned, best pBlast hit was provided in brackets]

[2] other than hypothetical

## Figures

**Figure 1. Whole genome synteny of *Babesia canis*, *B. bovis,* and *B. microti*.** Assembled *B. canis* contigs (top arc) were arranged based on synteny to related *Babesia* species. Predicted arrangement of *B. canis* contigs on four chromosomes is indicated by colours of the lines linking conserved genes. Blood stage mRNA expression data for *B. canis* genes is included (line plot); gene density is colour coded and represented as number of genes per 10kb (heatmap). Chromosomal distribution of *B. canis* VESA gene models is indicated with red dots.

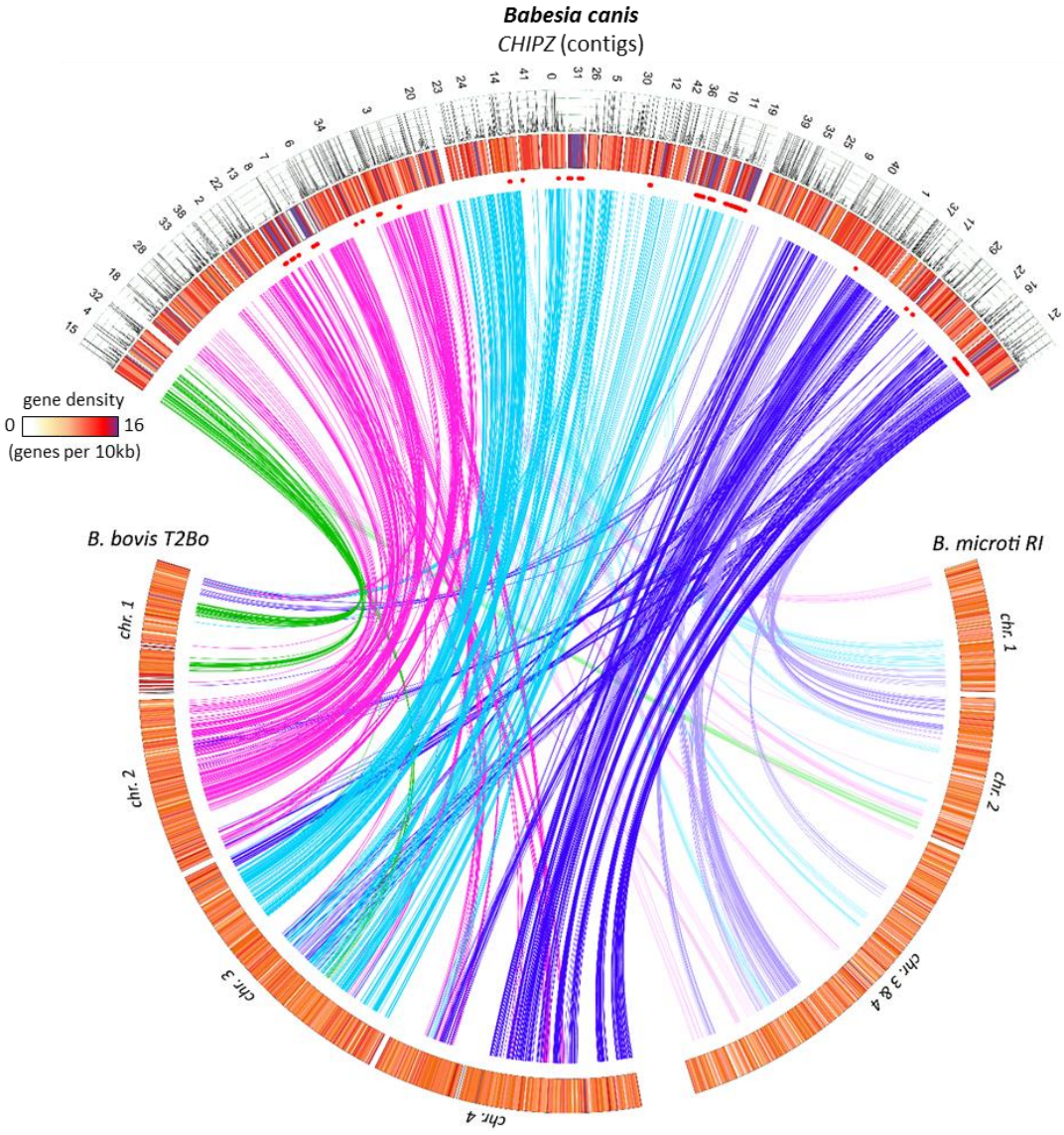


**Figure 2. Comparative analyses of the *Babesia canis* genome.** Gene clusters among different apicomplexan parasites as identified by OrthoMCL. (A) Comparison of orthologous clusters in four *Babesia* species and (B) *B. canis* and representative Apicomplexa species. The number of orthologous groups is indicated in the intersections. The total number of gene-models and clusters for each species is shown in brackets. Orthologous gene clusters in (A) are divided into three categories (piechart colour code: ■ multigene family; ■ genes with annotations; ■ hypothetical genes).

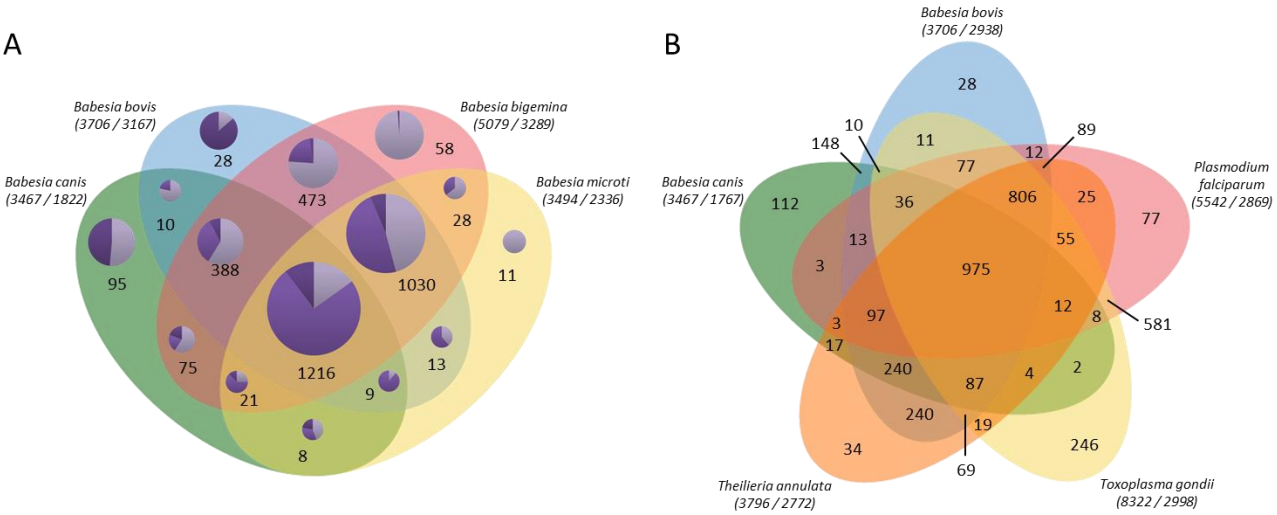



**Figure 3. Conserved apicomplexa-specific exported protein families in *Babesia canis*.** Individually identified gene models were assigned to gene families of phylum-specific exported proteins; bar colours in the graph indicate the predicted route of secretion and/or type of membrane anchoring. CSP: canonical signal peptide; aSP: alternative (non-classical) secretory pathway; TM: transmembrane domain; GPI: predicted glycophosphatidylinositol anchor.

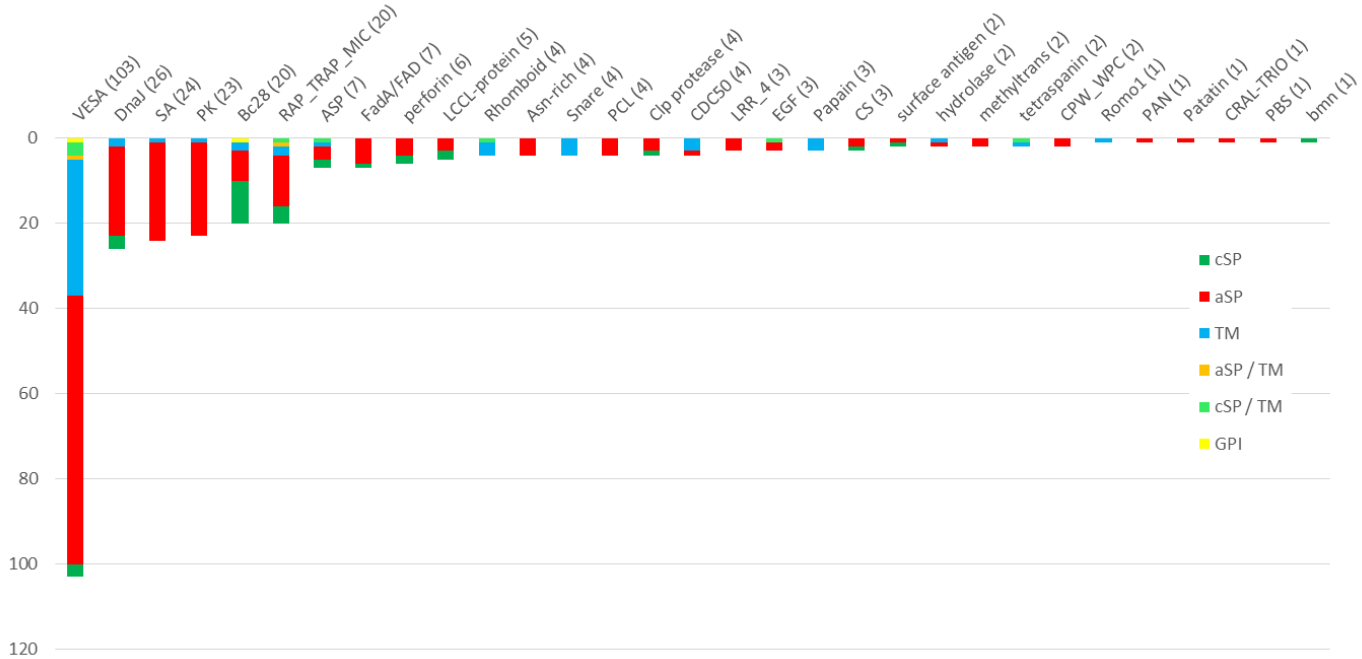


**Figure 4. Transcript abundance of exported *Babesia canis* proteins.** Collated (A) and individually ordered (B-F) RNAseq data plots of members of 12 gene families, showing FPKM values for 103 gene models from the VESA- (B), 26 gene models from DnaJ- (C), 24 gene models from the SA- (D), 23 gene model for the PK- (E), and 20 gene models from the Bc28-multigene family. FPKM: Fragments Per Kilobase of exon model per Million mapped reads.

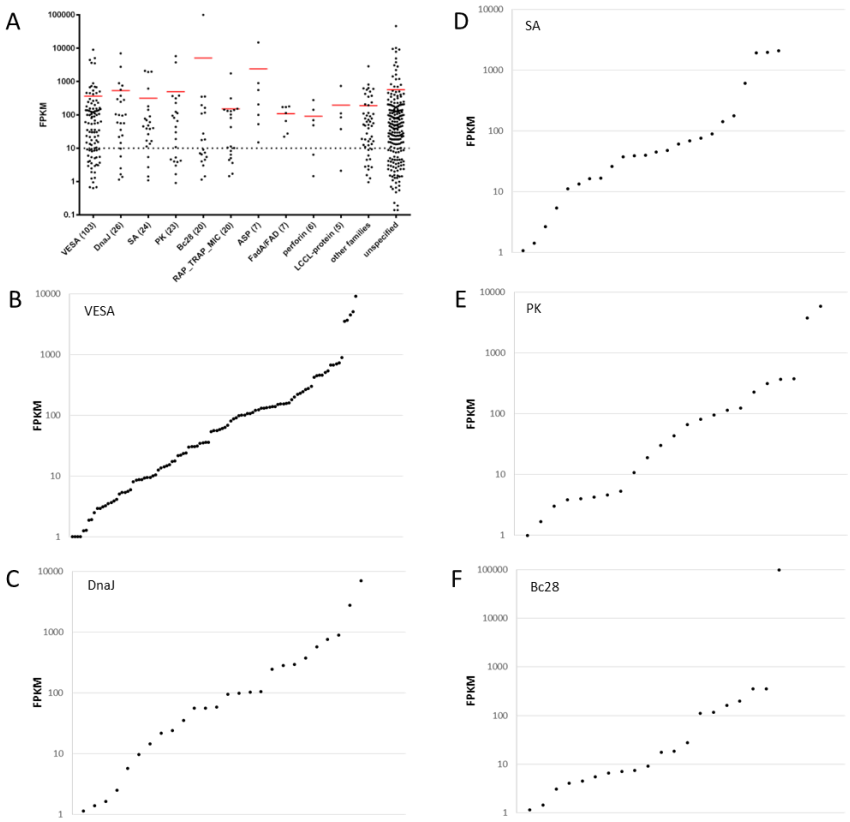
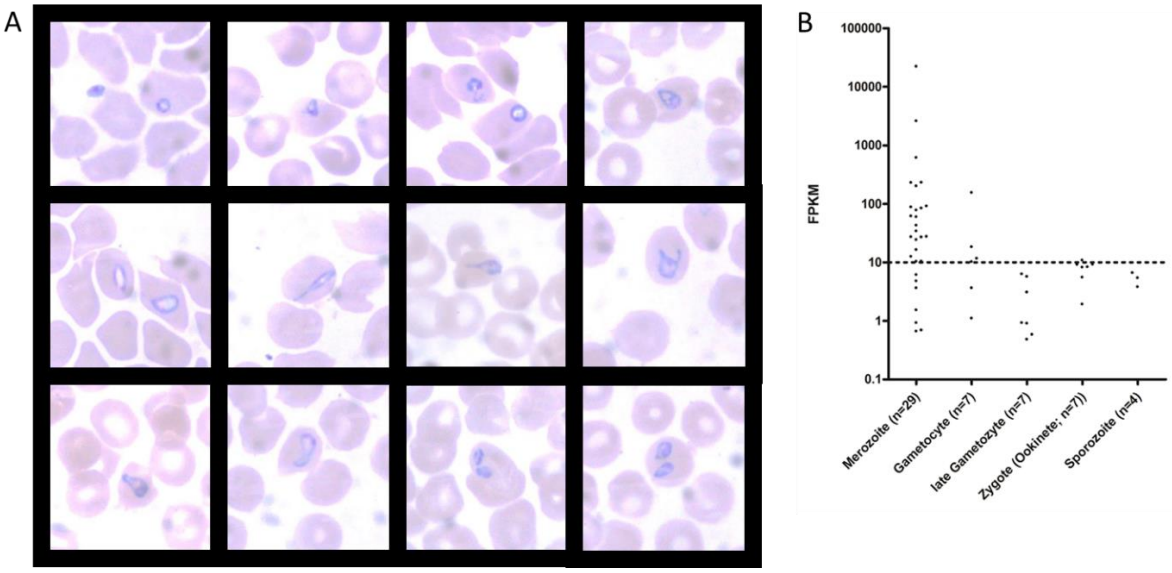



**Figure 5. *Babesia canis* blood stages.** (A) Representative Giemsa-stained blood smears from an experimentally infected dog with severe shock-like clinical signs showing typical pleiomorphic morphological appearance of *B. canis* parasites (Parasitaemia of 1.5% infected erythrocytes). (B) Transcriptome analysis of *B. canis* homologs of validated *Plasmodium* spp. stage-specific genes in a blood sample collected from an experimentally infected splenectomized dog with clinical signs consistent with acute canine babesiosis.



**Supporting information**

*Supporting Information will be made available with the final version of the publication:*

**Supplemental Data S1.** OrthoMCL cluster list. Gene-clusters which are shared between 4 annotated *Babesia* species (*B. canis*, *B. bovis*, *B. bigemina*, *B. microti*) and gene-clusters which are shared between 5 annotated Apicomplexa species (*B. canis*, *B. bovis*, *Plasmodium falciparum*, *Toxoplasma gondii*, *Theileria annulata*).

**Supplemental Data S2.** OrthoMCL cluster list. Gene-clusters which are specific for assembled *Babesia* species (*B. bovis*, *B. bigemina*, *B. microti*) other than *B. canis*. (These gene-clusters are not present in the *B. canis* annotation).

**Supplemental Data S3.** Predicted exportome of *Babesia canis* CHIPZ strain (Hungary) based on in silico analysis.

**Supplemental Data S4.** Transcriptome analysis of stage specific genes in the *B. canis* genome. Annotated genes were blasted against a list of known and approved *Plasmodium* spp. stage-specific genes and FPKM values from the *B. canis* RNA-seq were assigned.

**Supplemental Data S5.** OrthoMCL cluster list. Gene-clusters which are specific for *Babesia canis* as compared to other assembled *Babesia* species (*B. bovis*, *B. bigemina*, *B. microti*).

**Supplemental document S1.** CEGMA completeness and completeness relative to *Toxoplasma gondii* core eukaryotic gene families in selected apicomplexan species.

**Supplemental document S2.** Genome overview.

**Supplemental methods.** Isolation of genomic DNA, library preparation and genome sequencing; Genome assembly and annotation; Analysis of genome completeness; RNA preparation, wet lab transcriptomics, and transcriptomic data analysis; Functional annotation and characterization; Prediction of exported gene models; Samples for proteomic studies; Mass Spectrometry and protein identification; References

**Supplemental Figure S1.** Selection criteria for the identification of the *Babesia canis* exportome.

**Supplemental Figure S2.** Venn diagram of specific *B. canis* exported proteins.

**Supplemental Figure S3.** Parasitaemia, prognostic markers, and acute phase response in three dogs experimentally infected with *B. canis*.

**Supplemental Figure S4.** Sequence similarities of highly *B. canis* specific gene families. Screenshots from ClustalΩ alignments of proteins which clustered in OrthoMCL analysis.

## 2.2. Prognostic markers in acute *Babesia canis* Infections

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**Abstract**

**Background:** Canine babesiosis, caused by *Babesia canis*, is a prevalent and clinically relevant disease in Europe. Severe acute babesiosis is characterized by a high mortality but prognosis is not always correlated with clinical signs, nor with the level of parasitemia.

**Objective:** This study evaluated prognostic markers associated with poor outcomes in acute *Babesia canis* infections.

**Animals and Methods:** We compared the results of routine laboratory profiles, hand-held lactate and glucose analyzer, and the acute phase response in 2 groups of naturally-infected dogs (7 survivors and 8 non-survivors). Samples were collected at the time of first admission and before any treatment. Subsequently, the course of prognostic markers was followed in 3 dogs experimentally inoculated with *B. canis*.

**Results:** Non-survivors showed significantly higher concentrations of lactate, triglycerides and phosphate and lower hematocrit, leukocyte counts, total serum protein concentrations, and thrombocyte counts when compared to survivors. All non-survivors (8/8) had hyperlactatemia, whereas most survivors (6/7) had values within the reference range. All survivors had leucocyte counts within the reference range, unlike the non-survivors, which showed leukopenia. During the course of acute babesiosis, the variables serum lactate, triglyceride, and phosphate concentrations, and thrombocyte count did exceed a prognostic threshold only during acute crisis.

**Conclusions and clinical importance:** Poor outcome in acute *B. canis* infection is indicated by changes in the laboratory profile. Intensive care should be considered for dogs presenting with moderate anemia, severe thrombocytopenia, mild to moderate leukopenia, hyperlactatemia, moderately increased serum phosphate, and triglyceride concentrations, and moderately decreased total serum protein concentrations.

***Keywords*:** biomarker, canine babesiosis, dog, outcome,

**Abbreviations:** ALT: alanine aminotransferase; AP: alkaline phosphatase; APP: acute phase protein; AST: aspartate aminotransferase; AUC: area under the curve; BW: body weight; CRP: C-reactive protein; DIC: disseminated intravascular coagulopathy; EDTA: ethylenediaminetetraacetic acid; hpi: hours post infection; IFAT: immune fluorescence antibody test; IQR: interquartile range; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; PCR: polymerase chain reaction; RBC: red blood cell; ROC: receiver operation characteristic; SAA: serum amyloid A; WBC: white blood cell

## Introduction

Canine babesiosis is a tick-borne disease caused by apicomplexan hemoprotozoan parasites. The 3 distinct large *Babesia* species, *B. canis*, *B. vogeli* and *B. rossi*, and the small *B. gibsoni*, *B. conradae* and *B. annae* have been characterized in dogs.[1-3] *Babesia canis* is the predominant and clinically relevant canine *Babesia* species in Europe [4] and infection typically is characterized by lethargy, apathy and pale mucous membranes. The disorder can manifest as a mild or severe form. The clinical signs of the severe form are variable and often related to an excessive inflammatory response syndrome associated with multiple organ dysfunction, shock and high mortality.[3,5]

Hematological abnormalities in natural and experimental *B. canis* infections include anemia, thrombocytopenia and inconsistent leukocyte abnormalities such as leukocytosis, leukopenia, neutrophilia, neutropenia, and eosinophilia. The most common abnormalities in the serum biochemical profile are increases in the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), hyperbilirubinemia, hypoalbuminemia, and electrolyte and acid-base abnormalities.[6-10] Babesiosis in dogs affects primary and secondary blood coagulation and can induce disseminated intravascular coagulopathy (DIC).[11,12] Furthermore, a systemic inflammatory response syndrome has been described in acute *B. canis* infection characterized by an acute phase response.[12-14]

The clinical manifestations of acute babesiosis are not always proportional to the degree of anemia, and are not correlated with the level of parasitemia, which often remains below 1%.[1,6,15] Hence, besides mechanical erythrocyte damage, other pathophysiological mechanisms have been proposed to contribute to hemolysis, such as toxic hemolytic factors and immune-mediated destruction of erythrocytes.[16] Furthermore, disease severity cannot be readily explained as a consequence of hemolysis alone, which often is mild to moderate in acute infections.[6] Severe complications of acute *Babesia* infections have been described such as hemolytic and septic shock, acute renal failure, multiple organ dysfunction syndrome, and other complications.[17,18]

The clinical outcome of a *B. canis* infection is influenced by many factors and the primary pathophysiological mechanisms of babesiosis in dogs remain unclear.[19,20] In canine *B. rossi* infections, poor prognosis and mortality are associated with hyperlactatemia, hypoglycemia, clinically compromised circulation, high parasite load, increased serum cortisol concentrations, and signs of consumptive coagulopathy.[21-25] Accordingly in *B. canis* infections, an excessive inflammatory response with increased concentrations of fibrinogen, C-reactive protein (CRP) and secreted intracellular adhesion molecule-1 (sICAM-1) from erythrocytes, and thrombocytopenia have been associated with poor outcome.[19] Furthermore, an increase in lipid mediators has been shown to be associated with severe complications such as development of systemic inflammatory response syndrome and multiple organ dysfunction.[5,26]

We aimed to evaluate routine laboratory and rapid in-clinic laboratory tests for their applicability as prognostic markers associated with poor outcome in acute *B. canis* infections in dogs.

## Material & Methods

*Animals*

*Naturally-infected animals*. The prognostic potential of different laboratory tests was evaluated in 15 naturally-infected animals, of which 12 dogs were presented to the Clinic for Small Animal Internal Medicine at the Vetsuisse Faculty, University of Zurich, and 3 dogs to private veterinary practices in Switzerland in the years 2011 to 2013. Inclusion criteria were the presence of acute clinical signs consistent with canine babesiosis at admission and the identification of large *Babesia* species by microscopic evaluation of Giemsa-stained blood smears. In each dog, *B. canis* diagnosis was confirmed by PCR [27] and direct sequencing of the amplicons.[a] At time of admission, blood samples were collected, and all animals were treated with antibabesial therapy (a single dose of 3 to 6 mg/kg body weight [BW] imidocarb diproprionate IM or combined with 10 mg/kg BW doxycycline PO every 12 hours for at least 10 days, and a second dose of imidocarb diproprionate after 14 days). Dogs were enrolled whenever inclusion criteria were met and adequate samples were available. The animals were categorized into 2 groups according to clinical outcome, which was defined as survival (survivor, n=7 dogs) or death (non-survivor, n=8 dogs). Six of the non-survivors died spontaneously within 24 hours of admission and 2 dogs had to be euthanatized within 48 hours because ofclinical deterioration within 48 hours. Survivors were considered to be cured based on the absence of parasites 14 days after first admission on evaluation of Giemsa-stained blood smears and PCR.

*Experimentally-infected animals.* The course of laboratory test results was evaluated in experimentally-infected animals. Three facility-housed adult beagles (of which 1 was 4 years and 2 were 6 years old) were inoculated IV with approximately 1 x $10^6$ parasitized erythrocytes from an isolate stored in liquid nitrogen. The parasite isolate originated from a naturally-infected Bernese mountain dog from

Switzerland that had travelled to Hungary. The experiments were terminated at the very first signs of acute crisis (which was defined as weak pulse, shallow breathing, somnolence, and any clinical signs of acute shock or central nervous depression). Experiments with dogs were conducted according to Swiss animal rights and regulations standards and approved by the Cantonal Veterinary Office of Zurich (permission number 122/2012) before the study.

*Samples*

Venous blood samples from the naturally-infected dogs were collected into tubes with and without ethylenediaminetetraacetic acid (EDTA) at the time of first admission and before any treatment. Serum and EDTA-preserved blood samples were collected through an indwelling catheter from the experimentally-inoculated dogs at different times. Additionally, citrated plasma samples were collected from these dogs at the end of the experiments.

*Analysis of blood samples*

Parasitemia was expressed as the percentage of infected erythrocytes in Giemsa-stained blood smears by manually scanning at least 5000 erythrocytes. Exposure to *Ehrlichia canis* and *Anaplasma phagocytophilum* was tested by an immuno-fluorescence antibody test (IFAT).[b,c] Complete blood cell counts were performed using EDTA-anticoagulated blood in an automated analyzer.[d] Hematological analysis included total white blood cell (WBC), thrombocyte and red blood cell (RBC) counts and RBC indices. Serum biochemical profiles were performed using an automated analyzer.[e] Laboratory reference intervals are stated as 5% and 95% quantiles. Portable hand-held devices for rapid in-clinic testing were used to measure concentrations of lactate[f] and glucose[g] immediately in freshly collected EDTA samples.[28,29] Serum CRP concentration was determined using a canine-specific immunoturbidimetric assay[h] and serum amyloid A (SAA) concentration was measured using a latex agglutination turbimetric immunoassay on an automated analyzer.[e,i] In the citrated samples from the experimentally infected animals fibrinogen concentrations were measured using the Clauss method and a semi-automated coagulometer.[k] D-dimer concentrations were measured on an automated analyzer.[e,l]

*Statistical analysis*

Results of the 2 groups (survivor and non-survivor) of naturally-infected dogs were compared by the Mann-Whitney U test. The initially significant variables then were analyzed with receiver operator characteristic (ROC) curves for which the area under the curve (AUC) was calculated. The ROC analysis was used for determining a prognostic cut-off value for best differentiating between survivors and non-survivors with a maximal Youden's index.[30,31] If the cut-off value fell within the normal reference

range, it was set at the corresponding border of the reference. Statistical analyses were performed using a statistical software package.[m] A p-value < 0.05 was considered statistically significant. The hematological and serum biochemical profiles from the samples collected at private practices were excluded from the analysis because these variables were measured with other analytical instruments. Hence, for these variables 6 survivors and 6 non-survivors were included. For parasitemia, variables from hand-held devices, and the acute phase response, all of the naturally-infected dogs were included in the analysis (7 survivors and 8 non-survivors). Graphs were generated using Graph Pad.[n]

**Results**

At admission all of the naturally-infected dogs had diverse clinical signs consistent with canine babesiosis including lethargy (all 15 dogs), pale mucous membranes (all 15 dogs), pigmenturia (10 of 15), icterus (6 of 15), pyrexia (5 of 15), anorexia (4 of 15), vomiting (4 of 15), "water hammer" pulse (4 of 15), and epistaxis (3 of 15). Although *Babesia* infection was assumed and antibabesial treatment initiated shortly after admission, 8 of the 15 dogs died or had to be euthanatized within 2 days of admission. All of the dogs were positive for *B. canis* in Giemsa-stained blood smears and by PCR, and none of these dogs reacted serologically to *E. canis* or *A. phagocytophilum* on IFAT. Data on characteristics of the individual dogs (animal description, travel history, and clinical signs) are summarized in supplemental file 1. No statistical difference in age, sex, and clinical signs was identified between survivors and non-survivors.

The parasitemia ranged between 0.5 and 3.1% (median, 1.2%; interquartile range [IQR], 0.83-1.63), but no statistical difference was identified in the level of parasitemia between the survivors and non-survivors. Results of laboratory findings as well as comparison between outcome groups are summarized in Table 1. In both groups of dogs, mild to moderate normochromic normocytic non-regenerative anemia, mild to severe hyperbilirubinemia, mild to moderate azotemia, mild to moderate hypoalbuminemia, mildly increased alkaline phosphatase (AP) activity, moderate to severe hyponatremia, moderate hypocalcaemia and a mild to moderate increase in CRP concentration were observed commonly. Non-survivors had significantly higher concentrations of lactate ($p<0.001$), triglycerides ($p<0.01$), and phosphate ($p<0.05$) and significantly lower hematocrit ($p<0.05$), WBC counts ($p<0.01$), total serum protein concentrations ($p<0.05$), and thrombocyte counts ($p<0.05$) than survivors.

These 7 initially identified prognostic factors were further analyzed by ROC analysis (Table 2). Of all variables studied, lactate concentrations and WBC counts showed the best prognostic sensitivity and specificity (both 100%) to differentiate between survivors and non-survivors. Figure 1 illustrates the significant prognostic variables for dogs in the 2 outcome groups. All non-survivors (8 of 8) had moderate to severe hyperlactatemia (median, 8.35 mmol/L; IQR, 7.18-9.13) whereas most survivors (6

of 7) had concentrations within the reference range (median, 1.6 mmol/L; IQR, 1.05-2.3). The WBC counts for all of the survivors (6 of 6) were within the reference range (median, 6.85 $x10^3$/µL; IQR, 6.03-8.2) unlike the group of non-survivors, which had mild to moderate leukopenia (6 of 6; median, 2.65 $x10^3$/µL; IQR, 1.7-3.53).

The course of the prognostic variable, parasitemia and the acute phase response was followed in the 3 dogs experimentally inoculated with *B. canis*. The 3 infected dogs became lethargic and showed signs of hemolysis (pale mucous membranes and pigmenturia) 105, 120, and 119 hours post-inoculation (on days 4 to 5), respectively. They had a low grade parasitemia with a maximum of 1.75% of the erythrocytes infected at the end of the experiment, and during the course 2 of the 3 dogs had episodes of pyrexia (Fig. 2a). An acute phase response could be observed with a moderate increase in CRP concentration and a moderate decrease in serum albumin concentration (Fig. 2b), whereas SAA concentrations remained below the diagnostic limit (data not shown). Follow-up of prognostic markers is shown in Fig. 3. A decrease in the hematological variables leukocytes, thrombocytes, and hematocrit was found before the identification of parasites in stained blood smears, and resulted in moderate leukopenia, severe thrombocytopenia and decreased hematocrit. In general, mild to moderate anemia was observed. Changes in lactate, triglyceride, and phosphate concentrations corresponded to the first appearance of parasites, and they only exceeded the prognostic threshold at the first observation of acute crisis. In addition, thrombocytopenia was a common finding and platelet counts exceeded the prognostic threshold toward the end of the experiment. Total serum protein concentrations also decreased over time but passed the threshold only in two of the three dogs before first signs of an acute breakdown. At the end of the experiment the three dogs showed mildly increased levels of fibrinogen of 2.6 g/L, 3.8 g/L, and 3.2 g/L (reference range, 1.0 – 2.5 g/L), and D-dimer concentrations of 0.26 mg/L, 0.44 mg/L, and 0.92 mg/L (reference range, <0.4 mg/L), respectively.

**Discussion**

In this study, several variables were shown to be associated with poor outcome in acute *Babesia canis* infections. By including 2 rapid in-clinic tests, standard hematological and biochemical variables, and acute phase proteins, we found the variables lactate, WBC, triglycerides, phosphate, thrombocytes, total serum protein, and hematocrit to be significant prognostic markers. Thus, non-survivors at admission had more severe anemia, leukopenia and thrombocytopenia in addition to alterations in their serum biochemical profile results.

Lactate concentrations were significantly lower in survivors and showed a clear difference from the non-survivors. This finding is similar to what is observed in dogs infected with *Babesia rossi*, the agent of severe canine babesiosis in South Africa, where serum lactate concentration is used for post-treatment monitoring, [21] and high blood lactate concentrations correlate with poor outcome.[32-34]

Nevertheless, the pathogenesis of hyperlactatemia in dogs with acute babesiosis is not well established, and it might not be caused by hypoxia as a consequence of anemia, which remains mild to moderate in most *B. canis* infected animals.[34] Hence, hypoxia in canine babesiosis may be the consequence of alterations in the macro- and micro-cirulation triggered by protozoal sepsis, hypotension, DIC, and systemic inflammatory response syndrome (SIRS), all of which are well known in *B. canis* infections [18,35]. Indeed, increased lactate concentrations have prognostic value in SIRS caused by various conditions.[36,37]

The second variable that clearly differentiate between the 2 studied groups was WBC count. Non-survivors had mild to moderate leukopenia, in contrast to the survivors with WBC counts in the reference range. Although the WBC count was a significant marker for outcome in our study, leukopenia was reported in 60% of mild cases of acute canine babesiosis.[17] Indeed, the WBC count fell below the prognostic cut-off before any clinical signs were observed in the experimentally-inoculated dogs. Severely affected dogs had mild to moderate neutropenia, with an overall degenerative tendency and lacking a left shift (see supplemental file 2). Furthermore, lymphopenia seems to be a hallmark of acute canine babesiosis.[17,19] A markedly increased serum cortisol concentration was found in dogs with lethal *B. rossi* infections, indicating a potential immuno-suppressed state in these animals, which also is indicated by an unexpected mild to moderate regenerative response of lymphocytes in dogs that survived.[24,38] Furthermore, studies in humans with acute malaria infections with *Plasmodium falciparum* and *P. vivax*, which are related to *Babesia* spp., identified mechanisms that could explain a depletion of lymphocytes from the peripheral blood by acute sequestration of the cells in the lymph nodes or other parts of the body or by immune cell exhaustion and abnormal cell death through parasite-induced apoptosis.[39,40] Similarly, toxic parasitic factors have been shown to be involved in canine *B. gibsoni* infection.[41]

Hemolytic anemia and thrombocytopenia are the most frequent abnormalities associated with a diagnosis of *B. canis* in naturally-infected dogs and thrombocytopenia usually is the most dramatic hematological abnormality in the course of babesiosis.[12,42-44] Our data indicate that severe thrombocytopenia is associated with poor outcome by a prognostic cut-off of 27,500 thrombocytes per µL, although a sensitivity and specificity of 83.3% for each indicates limited prognostic value. Presumably, several factors are involved in the origin of thrombocytopenia in canine babesiosis including increased platelet activation and consumption by a SIRS (hypercoagulable state), increased platelet sequestration and aggregation, and a decreased platelet production.[19,45,46] Comparable in *B. rossi* infections, poor outcome was associated with a consumptive coagulopathy, although even severe thrombocytopenia was not accompanied by apparent bleeding diathesis and hemorrhage.[25,47,48]

Increased phosphate concentrations often are associated with metabolic acidosis characterized by tissue hypoxia and high blood lactate concentrations, although the underlying mechanisms have not

been completely explained.[49] Hemorrhage, hypovolemia, and shock as cause or consequence of tissue hypo-perfusion could further explain changes in altered variables, including also azotemia and potential protein-losing nephropathy caused by hypoxic renal damage.[35] Complications related to hemolytic anemia, coagulation disorders and hypotension, SIRS, and secondary impaired renal function likely account for the severe outcome of the infection.[6,9,10,12] Furthermore, in other studies, acute respiratory distress syndrome, renal failure, immune-mediated hemolytic anemia, cerebral syndrome, and DIC were associated with increased mortality in acute *B. canis* infections.[17,50]

Acute phase proteins were used as prognostic factors for different inflammatory processes, [51] and an acute phase response also was observed in acute *B. canis* infections.[12-14,20] We measured the acute phase proteins CRP and SAA, because they are considered major APP in dogs [52] and are not significantly affected by hyperbilirubinemia, which is commonly present in acute babesiosis.[51] We found an increase in CRP before parasite detection as previously observed,[12] without any significant difference between the outcome groups. This finding is in accordance with findings in *B. rossi* infections in which no prognostic value for CRP concentrations was observed.[53] Furthermore, the SAA concentrations did not increase significantly in naturally- and experimentally-infected animals. This finding is in contrast to other observations of increased SAA concentrations in dogs with babesiosis on the day of admission.[14] As another indicator, serum albumin concentration could serve as a negative APP.[51] With the onset of acute infection, we observed a moderate decrease in serum albumin concentration and it had no prognostic relevance. Although differences between survivors and non-survivors were absent for an acute phase response, APP (among other variables) could serve as important variables for monitoring response to therapy.[14,54]

In the course of validating prognostic markers in 3 experimentally-inoculated dogs, we observed low grade parasitemia with a maximum of 1.75% of infected erythrocytes, which was comparable to the group of naturally-infected animals. Even in infections with serious clinical signs, low parasitemia is a common finding in *B.* canis infections.[1,6,12] The course in the infected dogs highlights the prognostic value of lactate, triglycerides, and phosphate concentrations, and thrombocyte counts, because these factors only crossed the prognostic threshold in an acute crisis. Referring to the ROC analysis of these variables, only lactate showed optimal characteristics. Therefore, any prognosis based on individual variables should be interpreted with caution.

Missing data about the course of disease before admission and the time point of infection in the naturally-infected dogs is a limitation of this study. Generally, practitioners inquire about the duration of illness and the appearance of the first clinical signs, and they can estimate the time of the infection in affected dogs. In this respect, the prognostic markers are helpful for guiding clinical decision making. To get an overall picture of individual cases, a systematic collection of clinical, laboratorial, and other individual factors must be emphasized. For example, in our cohort of infected dogs, circulatory

disturbances were detected in 4 relatively young dogs (7 month to approximately 3 years), of which 3 dogs died (see supplemental file 1). Such clinical variables could affect outcome in the laboratory test results and the likely progression of a patient's infection.[55] In any case, outcome depends on a rapid diagnosis and early treatment.

Mortality in the investigated group of dogs was higher as compared to an endemic area.[5] This finding reflects a typical situation for non-endemic areas such as Switzerland, where dogs became infected from local *Babesia* outbreaks or have traveled to an endemic area. These dogs likely never have had contact with the parasite and therefore did not develop partial immunity.[46,56] Nonetheless, findings on mortality rates should not be over interpreted, because of the small sample size. In our cohort, we included every possible case for which we could obtain comparable laboratory data.

Unfortunately, we did not have precise data about infection rates in dogs in Switzerland. However, during the sampling period, 2 indigenous outbreaks were reported in 44 dogs, of which 10 died.[57,58] Most indigenous cases in our cohort originated from these areas (4 survivors and 1 non-survivor) whereas 1 dog originated from Geneva, a known endemic region in Switzerland.[59] The remaining 9 infected dogs had a positive travel history. Information about infection rate in dogs in Switzerland that have travelled is rare. For example, from 2011 to 2013, the diagnostic unit of the Institute of Parasitology in Zurich (which offers a travel screening panel) identified 2.1% of 804 samples as positive on blood smears for large *Babesia* species (F. Grimm, personal communication). This observation is in agreement with observed cases in dogs in Germany that have travelled, with 3.7% (19/508) of animals positive for large *Babesia* spp. in Giemsa-stained blood or buffy coat smears.[60] Hence, to compensate for the small sample size, prognostic markers were cross-validated in the course of experimental babesiosis.

Although a significant prognostic marker is not necessarily clinically relevant, the pathophysiological reason for death would be of interest. With this in mind, additional studies should include post-mortem examination, and more prognostic factor studies should be conducted including other non-routine variables. The current study focused on rapid in-practice tests (e.g. lactate and glucose determined by hand-held analyzers) and routine laboratory variables, and the associated findings summarize the prognostic value of these variables. Nevertheless, additional research is needed to evaluate what additional evaluation and intensive care is needed for dogs with a poor prognosis. In this context, several markers have been demonstrated as good variables for follow-up and post-treatment monitoring after antibabesial therapy, such as APP, lactate, thrombocytes, and leukocytes.[13,14,21,43,54]

**Ethical standards and conflict of interest statement**

Animal experiments were carried out at the experimental units of the Vetsuisse Faculty at the University of Zurich after approval by the Cantonal Veterinary Office of Zurich (permission number 122/2012) according to Swiss animal rights and regulation standards.

The authors declare no conflict of interest.

**Footnotes**

[a] Synergene GmbH, Schlieren, Switzerland

[b] Mega Screen Fluoehrlichia c., MegaCor Diagnostik GmbH, Hörbranz, Austria

[c] *E. equi* FA substrate slide, VMRD, Inc. Pulma, Washington, USA

[d] Sysmex XT-2000iV, Sysmex Corporation, Kobe, Japan

[e] Cobas Integra 800, Roche Diagnostics, Rotkreuz, Switzerland

[f] Lactate Pro, Axon Lab AG, Baden, Switzerland

[g] Accu-Chek, Roche Diagnostics AG, Rotkreuz, Switzerland

[h] Gentian cCRP; Gentian AS, Moss, Norway

[i] LZ Test SAA; Eiken Chemical Co., Ltd., Tokyo, Japan

[k] STart 4, Roche Diagnostics AG, Rotkreuz, Switzerland

[l] Tina-quant D-Dimer Gen.2, Roche Diagnostics AG, Rotkreuz, Switzerland

[m] IBM SPSS statistics, 20.0.0, IBM Corp. Armonk, NY, USA

[n] Graph Pad Prism 4, Graph Pad Software, San Diego, USA

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## Tables

**Table 1.** Median values of various variables (minimum – maximum value) in dogs with naturally acute *Babesia canis* infections: a comparison between survivors and non-survivors.

| Variable (unit) | Reference range | Survivors | Non-survivors | p-value |
|---|---|---|---|---|
| Parasitemia (%) | | **1.2 (0.5 - 3.1)** | **1.25 (0.5 – 1.9)** | 1 |
| *Fast in-clinic variables* | | | | |
| Lactate (mmol/L) | < 2.5 | 1.6 (0.5 – 3.6) | **8.35 (4.3 – 11.8)** | **<0.001** |
| Glucose (mmol/L) | 3.9 – 6.7 | 4.8 (4.3 – 7.6) | 5.2 (3 – 11.9) | 1 |
| *Hematological variables* | | | | |
| Hematocrit (%) | 42 – 55 | **30 (25 – 40)** | **25.5 (17 – 28)** | **0.041** |
| Hemoglobin (g/dL) | 14.4 – 19.1 | **11.45 (9.2 – 14.2)** | **9.05 (7 – 18.2)** | 0.240 |
| RBC (x$10^6$6/µL) | 6.1 – 8.1 | **4.52 (3.95 – 6.2)** | **3.95 (3.05 – 5.39)** | 0.240 |
| MCH (pg) | 23 – 26 | 24.5 (22 – 29) | 23 (22 – 24) | 0.180 |
| MCHC (g/dL) | 34 -36 | 35.5 (34 – 36) | 35 (35- -39) | 0.937 |
| MCV (fL) | 64 – 73 | 67.5 (64 – 74) | 65 (58 – 67) | 0.093 |
| WBC (x$10^3$/µL) | 4.7 – 11.3 | 6.85 (5.1 – 9.2) | **2.65 (1.59 – 4.5)** | **0.002** |
| Thrombocytes (x$10^3$/µL) | 130 – 394 | **45 (17 – 190)** | **14.5 (6 – 41)** | **0.026** |
| Reticulocytes (%) | | 0.31 (0 – 0.81) | 0.53 (0.29 – 1.17) | 0.132 |
| *Biochemical variables* | | | | |
| Total bilirubin (µmol/L) | < 3.5 | **21.25 (4.7 – 85.3)** | **54.7 (19.7 – 221.8)** | 0.240 |
| Urea (mmol/L) | 3.8 – 9.4 | **12.65 (4.5 – 25.7)** | **30.45 (6.2 – 79.4)** | 0.065 |
| Creatinine (µmol/L) | 50 – 119 | 88 (75 – 133) | 84 (54 – 651) | 1 |
| Total protein (g/L) | 56 – 71 | 57 (40 – 64) | **44 (34 – 50**) | **0.026** |
| Albumin (g/L) | 29 – 37 | **26.5 (19 – 38)** | **24 (13 – 26)** | 0.240 |
| Cholesterol (mmol/L) | 3.5 – 8.6 | 6.25 (4.9 – 7.7) | 6.05 (2.7 – 9.3) | 0.589 |
| Triglycerides (mmol/L) | 0.4 – 1.5 | 0.85 (0.8 – 1.1) | **1.95 (0.9 – 3.5)** | **0.009** |
| Alkaline phosphatase (U/L) | 20 – 98 | **113.5 (74 – 184)** | **253.5 (61 – 358)** | 0.132 |
| ALT (U/L) | 20 – 93 | 47.5 (26 – 72) | 72.5 (30 – 96) | 0.132 |
| Sodium (mmol/L) | 152 – 159 | **144.5 (132 – 154)** | **142.5 (140-151)** | 0.485 |
| Potassium (mmol/L) | 4.3 – 5.3 | **4.2 (3.6 – 4.5)** | 4.4 (3.6 – 5) | 0.394 |
| Chloride (mmol/L) | 113-124 | 113.5 (97 – 115) | **109 (94 – 121)** | 0.485 |
| Calcium (mmol/L) | 2.4 – 2.8 | 2.43 (2.11 – 2.52) | **2.225 (2.1 – 2.55)** | 0.180 |
| Phosphate (mmol/L) | 1.0 – 1.6 | 1.36 (1.05 – 1.68) | **2.54 (1.39 – 3.08)** | **0.015** |
| *Acute phase response* | | | | |
| canine CRP (mg/L) | < 5 | **84.7 (3.3 – 169.8)** | **155.55 (22.5 – 232.8)** | 0.189 |
| SAA (mg/L) | < 2.19 | 0 (0 – 2.5) | 0 (0 – 1.1) | 0.536 |

**Table 2.** Results of the ROC analysis with prognostic cut-off values of significantly altered variables and respective sensitivity, specificity, area under the curve (AUC) and standard error (SE) associated with the outcome in *Babesia canis* infected dogs.

| Parameter (unit) | Prognostic cut-off value | Sensitivity (%) | Specificity (%) | AUC | SE |
|---|---|---|---|---|---|
| Lactate (mmol/L) | 3.95 | 100 | 100 | 1.00 | 0.00 |
| Hematocrit (%) | 28.5 | 66.7 | 100 | 0.86 | 0.11 |
| WBC (x$10^3$/µL) | 4.7[a] | 100 | 100 | 1.00 | 0.00 |
| Thrombocytes (x$10^3$/µL) | 27.5 | 83.3 | 83.3 | 0.89 | 0.10 |
| Total protein (g/L) | 50.5 | 83.3 | 100 | 0.88 | 0.12 |
| Triglycerides (mmol/L) | 1.5 | 83.3 | 100 | 0.94 | 0.07 |
| Phosphate (mmol/L) | 1.72 | 83.3 | 100 | 0.92 | 0.09 |

**[a] set at the border of the reference range (calculated cut-off at 4.8x$10^3$/µL)**

## Figures

**Fig. 1.** Prognostic markers in acute *Babesia canis* infected dogs. Median, and interquartile range for significant prognostic markers (p<0.05) recorded at admission in naturally infected dogs that did or did not survive an acute *B. canis* infection. Dots correspond to the data from individual dogs; the shaded grey areas represent the reference intervals. WBC: white blood cells

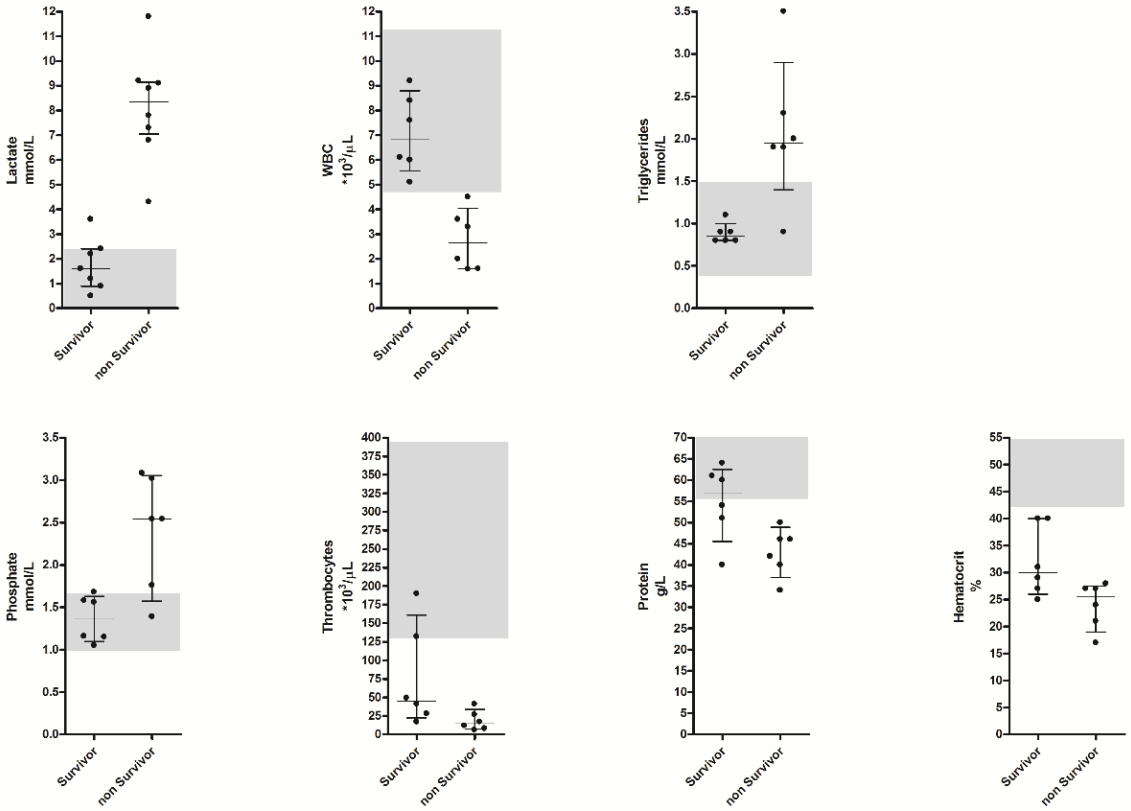


**Fig. 2.** Course of selected variables in 3 experimentally-infected dogs. Dog 1: solid line; dog 2: broken line; dog 3: dotted line. **a:** Body temperature (left y-axis) and parasitemia (right y-axes; lines with dots). The shaded grey area represents the reference interval for the body temperature. **b:** CRP (left y-axis) as a marker for positive acute phase response and albumin (right y-axis; lines with dots) as a marker for negative acute phase response. The shaded grey area represents the reference interval for albumin. hpi: hours post infection, CRP: C-reactive protein

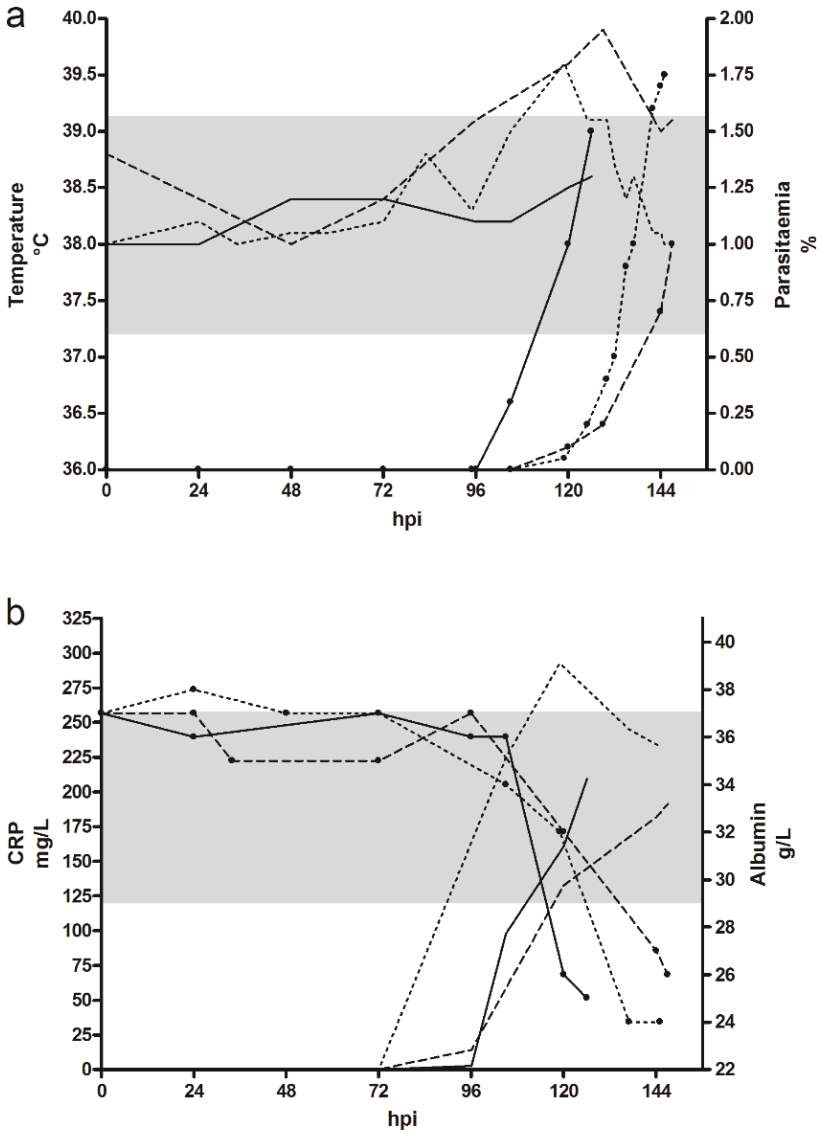
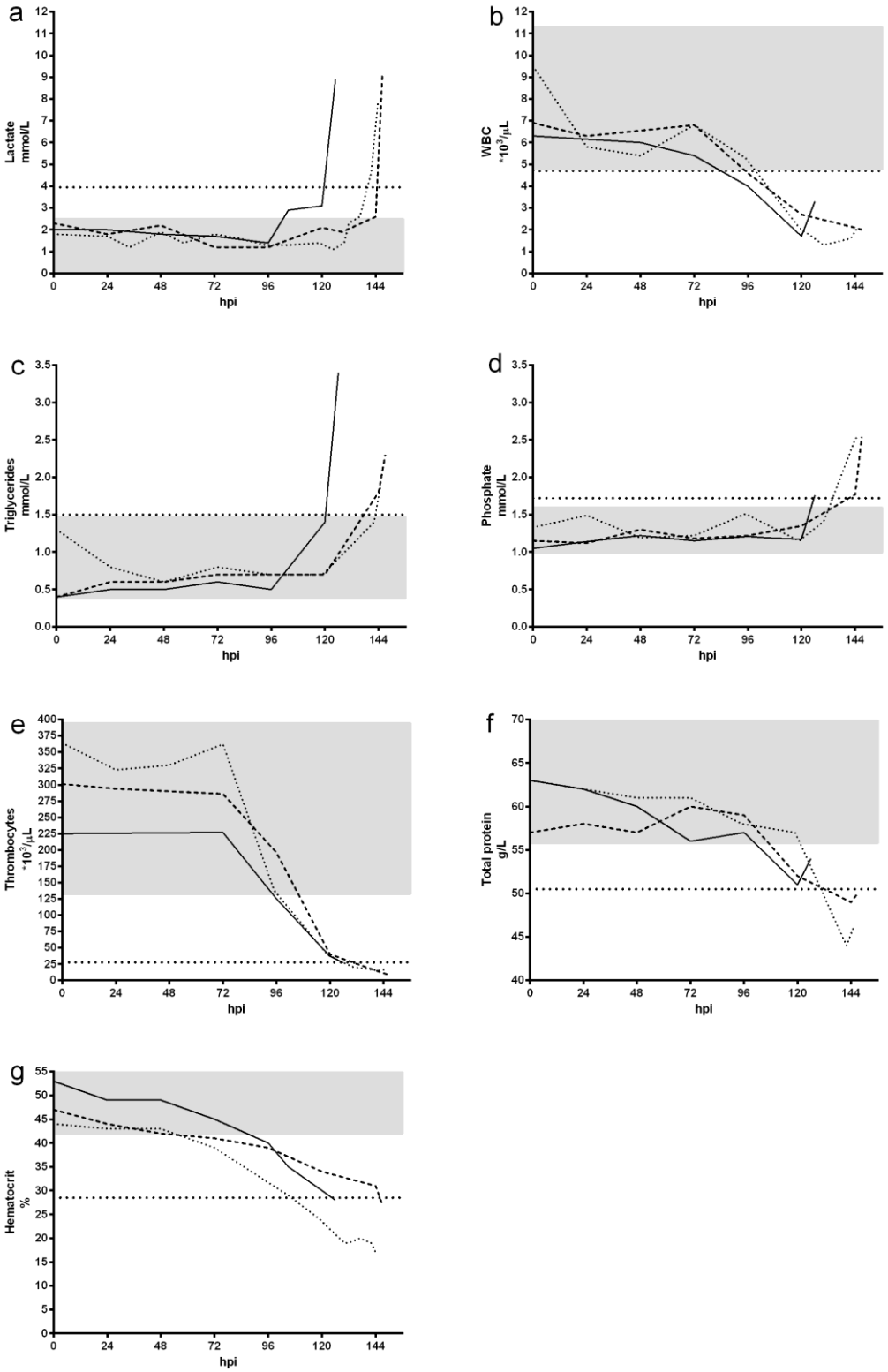


**Fig. 3.** Course of significant prognostic markers in 3 experimentally-infected dogs. Dog 1: solid line; dog 2: broken line; dog 3: dotted line. The shaded grey areas represent the reference intervals. The horizontal broken lines represent the corresponding prognostic cut-off. **a:** blood lactate, **b:** WBC, **c:** triglycerides, **d:** phosphate, **e:** thrombocyte count, **f:** total protein, **g:** hematocrit. hpi: hours post infection, WBC: white blood cells



**Supporting information**

*Additional Supporting Information may be found online with the publication in 'Supporting Information':*

**Supplemental file 1.** Characteristics of the individual dogs (animal description, travel history, and clinical signs).

**Supplemental file 2.** Data on differential WBC count. Differential WBC count in the course of three experimentally inoculated dogs, and in dogs that did or did not survive a naturally acquired acute *Babesia canis* infection.

## 2.3. Ticks on dogs and cats: a pet owner-based survey in a rural town in northeastern Switzerland

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**Abstract**

Changes in the endemic foci of tick populations and invasions of tick species to new areas have become evident in Europe, leading to changes in the epidemiology of tick-transmitted diseases. However, data about tick infestations of pet animals are limited. Following the recent identification of a new focus of canine babesiosis in northeastern Switzerland, we investigated the occurrence of tick vectors in this region by using a pet owner-based sampling strategy. All dog owners in a rural town were sent postal requests to send ticks from their dogs and cats over two consecutive years, beginning in April 2012. In total 3003 ticks were submitted for identification from 249 dogs (approximately 20% of the resident dog population) and from 117 cats. *Ixodes ricinus* was the most abundant species identified in 96.8% (n=2124) and 74.3% (n=601) of the individual samples submitted from dogs and cats, respectively. Two other tick species*, I. hexagonus* and *Dermacentor reticulatus*, were recorded on both host species, with host infestation prevalences below 2%. On cats (but not on dogs), as many as 24.0% (n=194) of the specimens were identified as a fourth tick species, *I. trianguliceps*. Overall, 93.5% of the ticks were adults (93.8% and 93.0% in dogs and cats), 4.4% nymphs (5.7% in dogs and 1% in cats) and 2% larvae (0.5% and 6.0% in dogs and cats), respectively. The highest infestation intensity was 49 *I. ricinus* ticks from an individual dog. However, 55.6% of the submissions from dogs and 24.8% from cats contained only one tick. This survey demonstrated that pet owners can contribute to a cost-effective tick surveillance and identified a new tick focus of *D. reticulatus*. The finding *of I. trianguliceps* exclusively on cats might be related to behavioural traits of the cats or to a more readily detection of these very small ticks during petting by their owners.

**Keywords**: dog; cat; *Ixodes ricinus*; *Ixodes trianguliceps*; *Ixodes hexagonus*; *Dermacentor reticulatus*.

## Introduction

Hard ticks (Ixodidae), frequently found on dogs and cats, bear the vector competence for a broad spectrum of pathogens of medical and veterinary significance (ESCCAP, 2012; Gray et al., 2009; Hillyard, 1996). In recent years, there have been changes in the endemic foci of tick populations, and invasions of tick species in central and northern Europe have become evident, causing changes in the epidemiology of tick-transmitted diseases (Beugnet and Marie, 2009; Jongejan and Uilenberg, 2004; Leschnik et al., 2002; Medlock and Jameson, 2010). For example, canine babesiosis is a severe and often life-threatening disease in dogs, caused by the protozoan *Babesia canis* which is transmitted by *Dermacentor reticulatus* (Halos et al., 2014). In Switzerland, there is a stable endemic focus in the western part of Lake Geneva (Jacquier, 1974; Pfister et al., 1993; Porchet et al., 2007), and four focal outbreaks in dogs, without a travel history, have recently been identified in eastern areas of the Swiss midplains (Sager et al., 2005; Schaarschmidt et al., 2013, 2006). We have identified a new focus in northeastern Switzerland, with 15 confirmed indigenous cases over the last 4 years (unpublished data). Therefore we aimed to investigate the occurrence of tick vectors in this region. Data on tick infestations of pet animals are rare, and it is presumed that different sampling strategies influence the composition of tick samples (Lavender and Oliver, 1996). Most published data on ticks of pet animals were obtained with specimens collected in veterinary practices (Claerebout et al., 2013; Földvári and Farkas, 2005; Nijhof et al., 2007; Ogden et al., 2000; Papazahariadou et al., 2003; Smith et al., 2011). Alternatively, ticks can be collected from the environment, by either experimentally walking dogs in the infested area or by flagging (Duscher et al., 2013; Jennett et al., 2013; Leschnik et al., 2012; Schorn et al., 2011).
Our study describes a systematic and cost-effective, pet owner-based sampling strategy to investigate tick occurrence on dogs and cats following an outbreak of canine babesiosis in a town surrounded by recreation areas in northeastern Switzerland.

## Material & Methods

*Study site*

The study was performed in the municipality of Rapperswil-Jona (population of approx. 26'000 inhabitants), which is located in the eastern part of Switzerland (47.233° N, 8.823° E) on the shores of the lake of Zurich. The altitude ranges between 407 m and 544 m above sea level. The vegetation around the town is predominantly characterised by deciduous woodland, bushes, agricultural areas and some rough grassland, rivers and marsh. The surrounding areas are popular recreation areas and attractive sites for dog walking.

*Tick sampling strategy and identification*

At the beginning of April 2012 and April 2013, letters were sent to all dog owners of the municipality (799 and 783 letters were sent in 2012 and 2013, respectively, to a total of 967 people). Their addresses were made available by the municipal administration, since all dogs (but not cats) in Switzerland are marked by a microchip and are registered (Swiss Federal Council directive No. 916.401). The dog owners were invited to collect ticks from their dogs and cats, store the ticks between adhesive tapes or in a suitable tube and send them to our laboratory. Furthermore, they were asked to provide data on the animals (dog/cat, age, sex, and breed) and on date and putative geographical site of tick exposure. The study lasted for two years and was terminated end of March 2014. Tick species, stage and gender were determined using standard keys (Estrada-Peña et al., 2004; Hillyard, 1996; Nosek and Sixl, 1972), and the ticks were stored individually in 70% ethanol at -20 °C.

*Tracking* D. reticulatus *in the environment*

To verify the presence of *D. reticulatus* foci, the owners of dogs infested with this species were contacted by telephone and asked where exactly they usually walk their dogs. Questing ticks along the common walking paths in the 3 identified distinct areas were collected by flagging the vegetation on four occasions (twice each in June and September 2012). The cotton flagging blanket was inspected every 20 m for the presence of ticks.

*DNA isolations and PCRs*

DNA from individual *D. reticulatus* ticks was extracted according to Wenk et al. (2012) using a mixer mill (Retsch®, MM 300). PCRs were performed using two sets of primers, both amplifying part of the *Babesia* spp. 18S rRNA gene as described (Casati et al., 2006; Hilpertshauser et al., 2006). To check whether amplifiable DNA was extracted, a tick-specific PCR targeting the mitochondrial 16S rDNA gene (Black and Piesman, 1994) was also performed. The latter PCR, combined with direct sequencing of the amplicons (Synergene GmbH, Schlieren, Switzerland) was also used to confirm tick species identification (Bown et al., 2006).

*Case definition and statistical analysis*

Every sample containing ticks was classified as an infestation, and co-infestations (when different tick species in the same sample were observed) were counted as different infestation events for each species. Thus, an individual animal could give rise to several infestation events, caused by infestations at different dates and/or by several tick species. The Pearson's $\chi^2$-test was used to analyse differences amongst infestation events in dogs and cats for each tick species, and for the number of ticks independent of species. Similarly, differences in life stages of the ticks received from dogs and cats

were analysed with respect to the total number of ticks per host animal. A p-value < 0.05 was considered as statistically significant. Statistical analysis was performed in R version 2.14.2 (R development Core Team, 2012).

**Results**

In total 967 dog owners were invited by letter twice (April 2012 and 2013) to send ticks from their dogs and cats. Data were collected for 2 years after the initiation of the study. Ticks were received from 18.6% and 16.5% of the dog owners in Year 1 and Year 2, respectively, and 20.7% and 19.1% of the dog population was covered (as an owner can have more than one dog). Ticks were submitted in every month of the two year study. A total of 3003 ticks (1719 and 1284 per study year) were submitted from 249 individual dogs and 117 individual cats. From 102 dogs and 11 cats, ticks were sent for both years of the investigation. Thus, samples were submitted from 184 and 167 dogs in Year 1 and Year 2 respectively, and from 64 cats in each of the two years. Overall, 521 and 360 infestation events for dogs and 124 and 86 for cats were recorded in the two years, respectively.

Data about the ticks from dogs and cats are summarised in table 1. *Ixodes ricinus* was the most abundant species representing 96.8% (n=2124) and 74.3% (n=601) of the ticks submitted from dogs and cats (statistically significant difference among the host species). Two other tick species, *I. hexagonus* and *D. reticulatus*, were recorded in both host species, with abundances below 2%. In cats, as many as 24.0% (n=194) of the specimens were identified as a fourth tick species, *I. trianguliceps*. The identity of two specimens of each developmental stages of this species was confirmed by PCR and sequencing. Of the ticks received, 93.8% and 93.0% were adult ticks isolated from dogs and cats, respectively. In contrast, the percentages of nymphs (total 4.4%; 5.7% in dogs and 1% in cats) and larvae (total 2%; 0.5% in dogs and 6.0% in cats) were significantly different between the two host species. Co-infestations were recorded on only 8 dogs with both *I. ricinus* and *D. reticulatus*. The number of ticks per infestation event is shown in Fig. 1. The highest intensity of infestation was 49 *I. ricinus* ticks from an individual dog, but 55.6% of the submissions from dogs contained only a single tick, whereas in cats this percentage was significantly lower (24.8%).

The presence of a local population of *D. reticulatus* was confirmed, by flagging along a 3 km long section, in one of the areas where infested dogs were regularly walked (Joner Allmeind, 47.226° N, 8.856° E). Thus, 5 female and 3 male *D. reticulatus* as well as 427 *I. ricinus* were collected. At least one *Dermacentor* specimen was obtained at any of the four collection dates (two each in June and September). All *Dermacentor* ticks received either from pet owners (n=50) or collected by flagging (n=8) were PCR-negative for *Babesia* spp.

**Discussion**

Our strategy to collect ticks in a newly identified focus of *Babesia* transmission consisted of systematically requesting residential dog owners to collect and send ticks from their pet dogs and cats. Thus, ticks from about 20% of the overall resident dog population were obtained. In the first eight months after sending the request letters, ticks were obtained from 20.2 and 18.7% of the dog population. In a British study covering the 8 months between March and October, 22.9% (810/3534) of individual dogs thoroughly inspected at veterinary practices were infested by ticks (Smith et al., 2011). Thus, the analogous infestations rates found in this previous study and our study confirm that our approach is suitable to obtain reasonable estimates of canine tick infestation. The slight decline of tick infestations observed in the second year of our study might be caused by the fact that some first year participants had improved tick prophylaxis and therefore did not send any ticks in the second study year, as they anecdotally reported to us. We are not aware of a report on the extent of tick prophylaxis on dogs in Switzerland, but a recent local study involving 29 dogs revealed that around 55% of the animals had received such treatment (Schaarschmidt et al., 2013).

Although ticks were submitted in every of the 24 study months, we did not attempt to analyse tick seasonality. Numbers of ticks peaked shortly after sending the letters to the animal owners once per year and then decreased over the year (data not shown).

As expected, the vast majority of the submitted ticks (93.5%) were adult stages, consistent with studies relying on tick collections by professionals from dogs presented in veterinary practices in Hungary (94.8% adult ticks; Földvári and Farkas, 2005) or Belgium (89.2%; Claerebout et al., 2013). Our sampling strategy of macroscopic examination by pet owners yielded a comparable number of smaller stages nymphs and larvae. In addition, cat owners detected numerous very small *I. trianguliceps* stages. These findings confirm the validity of our approach to collect representative samples of ticks from pets. Interestingly, whereas the percentage of developmental stages was comparable in dogs and cats, a significantly higher proportion of *I. ricinus* larvae were sent from cats. This could either be due to the fact that cats indeed are more infested by subadult ticks because of behavioural traits or because of a more intense interaction of animal owners with their cats (i.e. caressing cat owners detect small ticks more effectively).

In Switzerland, at least 18 different tick species are endemic, and another ten imported species have been reported at focal spots from wild and domestic animals (Aeschlimann and Papadopoulos, 1998). There is limited information available about the occurrence of these tick species on companion animals in Switzerland and neighbouring countries. Only a few of the indigenous tick species have been identified on dogs and cats in these countries. These are usually *I. ricinus* and *I. hexagonus*. Rarely, *D. reticulatus*, *D. marginatus*, *I. canisuga* and *Haemaphysalis concinna* have been reported, and occasionally the imported species *Rhipicephalus turanicus* and *R. sanguineus* (Beichel et al., 1996;

Capari et al., 2013; Dautel et al., 2006; Duscher et al., 2013; Földvári and Farkas, 2005; Leschnik et al., 2012; Nijhof et al., 2007; Pfister et al., 1993; Porchet et al., 2007; Sager et al., 2005; Schaarschmidt et al., 2013). In our study, 90.7% of the ticks were either *I. ricinus or I. hexagonus. D. reticulatus* accounted for around 1% of the infestations, with comparable rates on dogs and cats. Surprisingly, the second most abundant tick species on cats was *I. trianguliceps*. The nidicolous *I. trianguliceps*, which is known to occur in Switzerland (Aeschlimann and Papadopoulos, 1998), has not, to the best of our knowledge, previously been reported on pet animals. It is usually found on rodents (Burri et al., 2011; Jameson and Medlock, 2011; Mihalca et al., 2012). Interestingly, *I. trianguliceps* was found only on cats but not on dogs, suggesting an influence of the roaming and rodent-hunting behaviour of cats (but no corresponding information was acquired from the cat owners). Again, other studies investigating the tick fauna of cats in Europe have never identified *I. trianguliceps.* The abundant *I. ricinus* and *I. hexagonus*, have frequently been identified and occasionally *I. canisuga* and a single *I. ventalloi* (Beichel et al., 1996; Capari et al., 2013; Claerebout et al., 2013; Nijhof et al., 2007; Ogden et al., 2000). *Ixodes canisuga* and *I. trianguliceps* can clearly be distinguished by morphology (Hillyard, 1996), and we have genetically confirmed the identity of eight *I. trianguliceps* specimens. It remains unclear why the cats in this study are the first ever identified population which was frequently infested with *I. trianguliceps*.

A few ticks of *D. reticulatus*, the vector species of *B. canis*, were identified, and this newly identified focus of this tick species in eastern Switzerland was subsequently confirmed by collecting questing ticks in a marsh-like environment with open meadows, loose trees and intense solar radiation, which are tick habitats comparable to those from other reports (Dautel et al., 2006). *Dermacentor reticulatus* could be collected in the field even though the time points of flagging (June and September), chosen for organisational reasons, were not during its seasonal peak activity (in March/April and October/November). Specimens of this tick species were submitted by animal owners from April to June (both years) and in October and November (1st year) and in September (2nd year).

All *Dermacentor* ticks collected in this study were tested negative for the presence of *Babesia*-DNA. This is in contrast to 19 of 23 ticks collected from a few square meters in a recreational site in the area of another recent canine babesiosis outbreak in the central part of Switzerland (Schaarschmidt et al., 2013).

*Conclusion*

Our study yielded comparable infestation rates and tick species spectra as compared with studies using more laborious approaches. In addition, it revealed the presence of an unexpected and probably overlooked tick species on cats and allowed to identify environmental habitats of the tick vector of *B. canis*. The sampling strategy which involves animal owners is powerful and cost-effective.

Furthermore, improvement might be possible by providing stamped, addressed reply envelopes to participating animal owners.

**Competing interests**

The authors declare that no competing interests exist.

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## Table

**Table 1.** Ticks submitted from dogs and cats throughout the two study years.

| | **Total** (%ticks[b]) | **Adults** (%ticks[a]) | | **Nymphs** (%ticks[a]) | **Larvae** (%ticks[a]) |
|---|---|---|---|---|---|
| | | Female | Male | | |
| **Dog (n=249):** | | | | | |
| *I. ricinus* | 2124 (96.8) | 1671 (87.7) | 319 (15.0) | 122 (5.7) | 12 (0.6) |
| *I. hexagonus* | 26 (1.2) | 19 (73.1) | 7 (26.9) | 0 | 0 |
| *D. reticulatus* | 44 (2.0) | 36 (81.8) | 5 (11.4) | 3 (6.8) | 0 |
| Total | 2194 | 1726 (78.7) | 331 (15.1) | 125 (5.7) | 12 (0.5) |
| **Cat (n=117):** | | | | | |
| *I. ricinus* | 601 (74.3) | 478 (79.5) | 84 (14.0) | 6 (1.0) | 33 (5.5) |
| *I. hexagonus* | 8 (1.0) | 8 (100) | 0 | 0 | 0 |
| *I. trianguliceps* | 194 (24.0) | 153 (78.9) | 23 (11.9) | 2 (1.0) | 16 (8.2) |
| *D. reticulatus* | 6 (0.7) | 6 (100) | 0 | 0 | 0 |
| Total | 809 | 645 (79.7) | 107 (13.2) | 8 (1.0) | 49 (6.1) |
| **Total[c]:** | 3003 | 2371 (79.0) | 438 (14.6) | 133 (4.4) | 61 (2.0) |

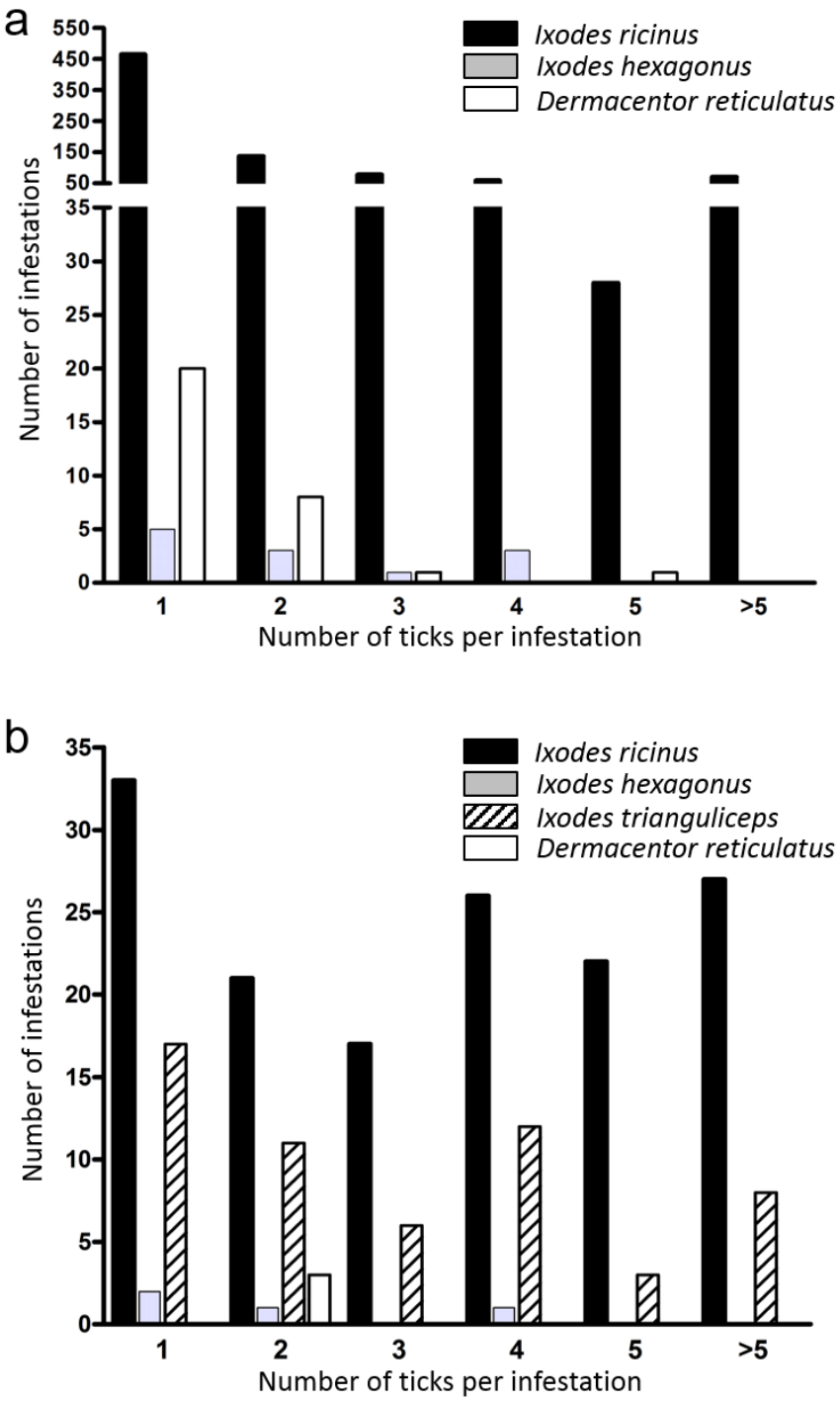
[a]percentage of ticks (per species)

[b]percentage of all ticks (per host)

[c]all species

## Figure

**Figure 1.** Number of ticks per infestation event in dogs (a) and cats (b).



# PART IV DISCUSSION & PERSPECTIVES

## 1. Discussion

### 1.1. General

*Babesia* in dogs has been recently characterized as a global emerging disease.[1] Although the epidemiology and pathophysiology of canine babesiosis has been studied extensively, many questions remain unanswered, and the underlying dynamics and molecular mechanisms are still poorly characterized. The present thesis addressed different aspects of this important canine disease contributing towards an enhanced understanding in pathological and molecular mechanisms of *B. canis* babesiosis, and provides data which can be translated for innovative intervention approaches.

In particular, I studied the occurrence of the vector-tick *Dermacentor reticulatus* in an emerging epidemiological focus for canine babesiosis (see Part III, Chapter 2.3.). Changes in the endemic foci of tick populations and invasion of tick species to new areas have become evident in Europe, leading to changes in the epidemiology of tick-transmitted diseases.[2-5] Investigations on tick species parasitizing companion animals demonstrated that pet owners can contribute to a cost-effective tick surveillance leading to the identification of a new tick focus of *D. reticulatus* in the eastern part of Switzerland. Besides, for the first time the presence of the "vole tick" *Ixodes trianguliceps* was reported exclusively on cats, explainable by the free-roaming and rodent hunting behavior of many cats.

Clinical cases of *B. canis* infections demonstrate a diversity of disease manifestations, depending on a variety of factors, including immune-response and age of the host, as well as the involved parasite strain.[6,7] By examination and evaluation of the outcome of clinical cases, a better understanding of the disease process and pathophysiological mechanisms are provided, resulting in the identification of novel routine clinical parameters and thus, to a better prognosis in severe acute infections (see Part III, Chapter 2.2.).

Recent work highlights the central role of parasite-specific exported proteins as potential virulence factors with a proposed role as pathogenicity determinants in acute *B. canis* infections (see Part III, Chapter 2.1.). As a prerequisite for transcriptomic- and proteomic analyzes, the whole genome of a virulent *B. canis* isolate from Hungary (*Bc*H-CHIPZ) was assembled and gene models were predicted and annotated. Global exportome analysis linked to shot-gun proteomics revealed secreted soluble and host membrane-bound proteins involved in the parasite-host interplay.

All these data and tools provide a basis for the design and evaluation of new intervention strategies (diagnosis, vaccination and treatment) and will contribute to an advanced understanding of basic concepts in infection biology.

## 1.2. Establishment of indigenous foci of canine babesiosis is dependent on the vector distribution

The incidence of canine babesiosis may vary considerably from one country to another and within different regions in a country, depending on the prevalent parasite and their specific vector. Hence, even though the climate in most European countries enables the propagation of *D. reticulatus*, the major vector tick for *Babesia canis*, the distribution of this species is highly focal.[4,8]

In Switzerland, there is a stable endemic focus of *B. canis* in the western part of the Lake Geneva with an expansion along the Jura mountains,[9-11] and four recent autochthonous outbreaks in the Swiss midplains have been published.[12-14] Additionally, some other indigenous cases in Switzerland have been reported (unpublished data). We have identified a new focus in northeastern Switzerland, with 15 confirmed cases reported from 2011 to 2014 (see figure IV-1). The presence of the vector tick could be demonstrated by the identification of a few *D. reticulatus* collected over two consecutive years. Different factors could be responsible for the establishment of a local transmission cycle, including the unusually warm and dry conditions during the recent outbreaks.[15] These outbreaks show that the resident canine population is immunological naïve resulting in acute and severe disease progressions and fatal cases.[7]

In the subsequent years, only few new infections have been observed in these dog populations (personal communication with local veterinarians). This reduced incidence rate can be attributed to the vector-biology, as the distribution of the vector tick *D. reticulatus* is highly focal.[8] For example, in one of the recent outbreaks in the Bernese 'Three Lakes region' (3 fatal cases from 9 infected dogs) infected ticks could be detected by flagging within a few square meters.[14] Accordingly, I identified one single spot with *D. reticulatus* ticks by flagging. An increased disease awareness led to improved diagnostic efforts and to a better tick control by highly effective spot-on applications and mechanical tick removal.[16] Similar dynamics of highly focal disease outbreaks in an apparent non-endemic region was described in the Netherlands with sporadic *B. canis* infections attributed to focal *D. reticulatus* foci and a subsequent decline of the incidence.[17]

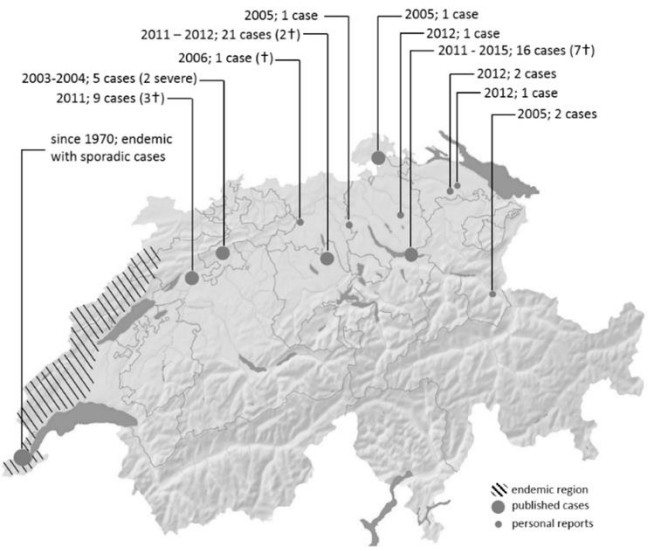



**Figure IV-1.** Geographical distribution of reported indigenous cases of *B. canis* in Switzerland until 2015. Reported year(s), in which infection(s) occurred and number of cases are documented. Fatal cases are highlighted (†).

In conclusion, monitoring vector and infection dynamics could be assisted by pet-owners resulting in a cost-effective tick surveillance. Furthermore, vector control is an important pillar in an integrated intervention approach.[18]

## 1.3. Clinical pathophysiology and prognostic markers: Are clinical signs host- or parasite induced?

Clinical *B. canis* babesiosis can manifest itself as mild self-limiting febrile illness or severe forms with high mortality.[1,19] The clinical outcome of *B. canis* infections are influenced by many factors and the primary pathophysiological mechanisms of canine babesiosis remain unclear.[20,21] Accordingly, prognosis is not correlated to clinical signs and the level of parasitemia, and a poor response of infusion therapy to the protozoal infection and shock is observed.[22-24] Measuring host factors as a response to the infection can assist in improving the understanding of pathophysiological mechanisms involved in host-parasitic interactions.

The general opinion underlying pathophysiological mechanisms for *B. canis* babesiosis are the uniform involvement of host inflammatory response to the parasite (SIRS and MODS, see Part II), rather than direct actions by the parasite itself.[21,25,26] Much of the observed clinical data can be attributed to an excessive immune-response (including also data from our studies, see Part III, Chapter 2.2.). Nevertheless, the pathogenesis of several significant factors present in dogs which died from acute babesiosis is not well established. This includes exceptional parameters such as the severe hyperlactatemia (hypoxic state), severe thrombocytopenia without apparent bleeding, diathesis and hemorrhage, and a potential immune-suppressed state by a lasting non-regenerative leukopenia, which could not be explained by a single host-response, although these clinical signs can correlate to SIRS. Hence, symptoms in complicated babesiosis are often referred as protozoal sepsis, as a consequence of dysregulation of the pro-inflammatory and anti-inflammatory mechanism during the acute phase of the disease including hypotension and end-organ failure.[24,27] It's likely that the multifactorial pathogenesis is triggered by the involvement of parasite-specific factors contributing to a severe host response, a mechanism well-described in bacterial sepsis by a range of virulence factors and toxins that interact with host cells dysregulating the host immune respons.[28]

Sequencing of the *B. canis* genome (designated as "BcH-CHIPZ") and bioinformatic approaches revealed that the parasite encodes many proteins predicted to be exported and presented to the host, therefore potentially involved in pathogenesis. The main exported protein families were the variant erythrocyte surface antigens (VESAs), DnaJ- (Hsp40) protein chaperones, the uncharacterized 'secreted antigen' family and members of the previously described Bc28-multigene family.[29,30] So far, the lack of efficient and effective culture techniques (and reverse genetics) for *B. canis* hampered the screening for virulence-factors. Therefore, we experimentally inoculated dogs aiming in characterizing parasite-specific proteins during acute disease. Our proteomic approach revealed 23 highly abundant proteins potentially involved in the parasite-host interplay (Part III, Chapter 2.1). Interestingly, proteins from different predicted gene families were present during the acute stage of the disease. This includes VESA, and Bc28 membrane proteins, and proteins from the 'secreted antigen' family. Despite the

presence of these potential virulence-factors, their role in pathogenicity has to be elucidated. An appropriate approach to address this question is missing, as direct *in vivo* injection of these proteins into dogs is difficult due to animal welfare considerations by the severe outcome of *B. canis* infections.

### 1.4. Models for alternative secretory pathways in *Babesia canis*

Most secreted proteins in eukaryotic organisms contain an internal signal peptide that direct their sorting to the endoplasmic reticulum (ER) and enable proteins to be exported to the extracellular space through the ER-Golgi secretory pathway. On the other hand, some cytoplasmic proteins that lack an ER-signal peptide have been shown to exit cells through ER-/Golgi independent pathways.[31] Many macromolecules with unconventional export pathways have been described to be of biomedical importance as crucial regulators of the immune response (including inflammatory cytokines in humans and animals), cell growth, differentiation and angiogenesis.[31]

By mining the *B. canis* exportome, a significant number of proteins in the predicted exportome (210 of 509 proteins) did not show a canonical hydrophobic N-terminal signal peptide for ER targeting. This finding is even more prominent by analyzing the *B. canis* protein families with apicomplexan homologues (see Figure 3 in Part III, Chapter 2.1). Although different models for unconventional protein secretion have been described,[31,32] the presence of these mechanisms in *B. canis* has yet to be shown, whereas initially potential miss-annotation of genes has to be excluded. Nevertheless, non-mutually exclusive scenarios for potentially unconventional protein secretion are described below in brief and schematically illustrated in figure IV-2, for some of which more evidence from the literature can be provided and are likely to be present in *B. canis*.

**Model 1** (pathway 2 in figure IV-2). ER-independent vesicular export of soluble cytosolic proteins by direct protein import to a secretory compartment. The *Babesia* micronemes or spherical bodies could represent possible secretory organelles for an alternative vesicular protein export.

**Model 2** (pathway 3 in figure IV-2). Small vesicle and/or exosome-based secretion system characterized by protein internalization into micro-vesicle and shedding from cell surface or by direct membrane-blebbing. Exosome-like particles have been observed in *P. falciparum* involved in host immune-modulation and inter-parasitic cellular communication [33,34] and could be also an important mechanism in non-classical protein export, as it has also been proposed for the protozoan parasite *Leishmania donovani,*[35] *Trypanosoma cruzi,*[36] and *Trichomonas vaginalis.*[37] Strikingly, the *B. canis* BcH-CHIPZ genome holds homologues of structural and functional proteins found in exosomes, including heat shock proteins (e.g. HSP70 and 90), tetraspanins and ALIX protein, TSG101/ESCRT protein, 14-3-3 protein, thioredoxine peroxidase, histones, and the RAB5, 7, 11, and RABGDI.[38-40]

**Model 3** (pathway 4 in figure IV-2). Protein export by translocation through membranes (parasite and RBC membrane). Non-vesicular export of proteins through different specific parasite-

induced translocons have been shown for *P. falciparum.*[41] Although export via multiple translocation steps may seem at first glance complex and energy-consuming, such a system could be more efficient than the establishment of a vesicular trafficking system through different membranes in a host cell (RBC) that is highly differentiated and has lost any trafficking mechanisms. The presence of a number of apicomplexan chaperones found in the host cell (including DnaJ/HSP40 chaperones in the *B. canis* exportome) might contribute to retain the secreted proteins in a translocation-competent form and could assist in the recruitment and translocation of specific proteins.[42,43]

**Model 4** (pathway 5 in figure IV-2). Protein export by membrane interaction of transmembrane domain-containing proteins. A potential mechanism is described for the *Leishmania* HASPB (hydrophilic acylated surface protein B) which is found associated with the outer leaflet of the plasma membrane only in the infectious stages of the parasite's lifecycle.[44,45] The protein is synthesized on free ribosomes in the cytoplasm and becomes both myristoylated and palmitoylated at its N-terminus (co- and posttranslational modification with saturated fatty acids chains), which is the molecular basis of how HASPB is anchored to the membrane.[46] Cytoplasmic HASPB is transferred to the outer leaflet of the Golgi membrane, from where it is transported to the plasma membrane via conventional vesicular transport. This process would insert HASPB into the inner leaflet of the plasma membrane. Presently, this model could not explain how a protein is then translocated across the membrane. A potential membrane-flipping step has to be involved.[32] Recently, alternative secretion in *P. falciparum* by posttranslational myristoylation and palmitoylation of proteins lacking an ER-signal peptide was demonstrated.[47] Furthermore, in other apicomplexans such as *T. gondii,* palmitoylation is highly prevalent.[48]

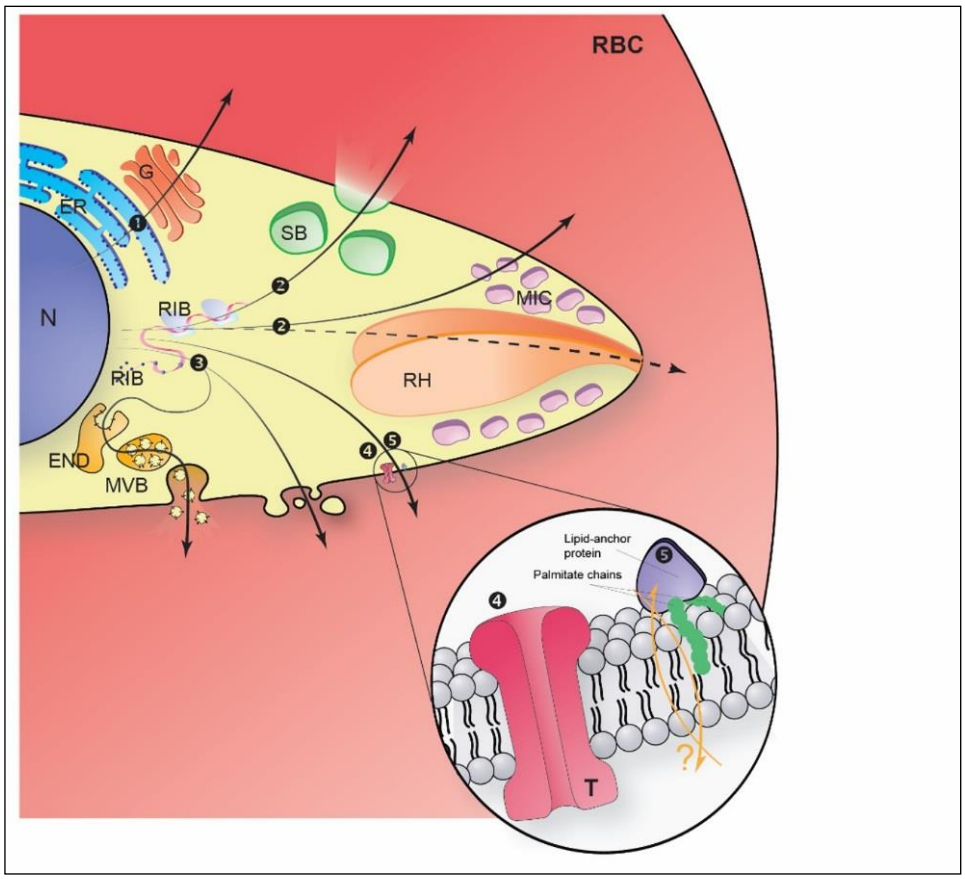



**Figure IV-2. Potential export routes of unconventional protein secretion in *Babesia canis***. Classical protein export pathways starting from translation at the rough endoplasmic reticulum followed by vesicular export including the Golgi network and secretory organelles (SB, MIC, RH) shown in the first pathway (1. Alternatively, unconventional export includes vesicular export by by-passing the ER to the parasite surface or the secretory organelles (2), by membrane-budding of microvesicles enclosing cytosolic proteins derived from free ribosomes (RIB) or by the export of exosome-like vesicles derived from early endosomes (END) and the fusion of multi-vesicular bodies (MVB) with the parasite plasma membrane (3). Further protein export includes the translocation of proteins through the plasma membrane (parasite and RBC) by parasite specific translocons (4). Membrane interaction/integration and export of co- and posttranslationally (e.g. palmitoylation and myristoylation) modified proteins include a so far undiscovered membrane flipping mechanism (5). END: early endosome; ER: endoplasmic reticulum: G: Golgi; MIC: micronemes; MVB: multi-vesicular body; N: nucleus; RBC: red blood cell; RH: rhoptry; RIB: free ribosomes; SB: spherical body; T: translocon.

## 2. Perspectives

Providing the whole genome of a virulent *B. canis* strain generated a powerful toolbox for further comparative studies and applications. Although robust *in vitro* culture techniques and reverse genetic approaches are not available to date, the here generated transcriptomic and proteomic data of blood-stage parasites give important clues about the pathophysiology of apicomplexan blood parasites. Therefore, the data could be translated to innovative intervention approaches, most needed novel diagnostic tools and effective vaccines.

## 2.1. Screening for diagnostic and vaccine candidates

Acute canine babesiosis represents an emergency situation and requires an early and accurate diagnosis for the immediate chemotherapy to increase the chance for survival.[7] In acute infections, diagnosis is routinely based on the direct demonstration of parasites in Giemsa-stained blood smears allowing a prompt diagnosis by experienced investigators. However, comparable to our studies, in subclinical-, latent-, but also in acute infections in animals with severe clinical symptoms, morphological diagnosis is aggravated by low parasitemia and a frequent absence of detectable parasites.[22,49,50] Therefore, innovative diagnostics are needed, for example as a rapid immunochromatographic test assay like it is applied for human malaria.[51,52] The gene models identified in this study and our *in silico* analyses provide many possible targets for further investigations. A potential list of proteins includes merozoite- and iRBC membrane antigens like proteins from the Bc28 multigene family,[30] apicomplexan conserved proteins with supposed species specificity like thrombospondin-related adhesive proteins,[53] and rhoptry-associated proteins,[54] as well as parasite aldolase, lactate dehydrogenase, and histidine rich proteins, known targets in rapid diagnostic tests for the diagnosis of malaria.[55] Hence, our studies may lead to the development of novel diagnostic tools.

Vaccines would be an ideal protective prevention method. However, the development of effective vaccines is hampered by the complexity and invasive biology of apicomplexan parasites which pass through multiple developmental stages, each stage expressing hundreds of unique antigens including further clonal variations. In fact, vaccine candidates against any apicomplexan parasite with this breadth of targets show limited vaccine's efficacy. Accordingly, the most prominent example is the commercialized human malaria vaccine 'RTS,S' (Mosquirix®, GlaxoSmithKline, UK) with 26% and 36% efficacy in infants and children, respectively.[56] Furthermore, for *B. canis* a vaccine formulation is on the market (Pirodog®, Merial, France). This crude formulation based on soluble parasite antigens (SPA) from infected blood culture only protects against severe clinical manifestations in some *B. canis* strains.[57] Adversely, uncharacterized crude protein fractions could include potential immune-modulatory proteins which minimize immune-stimulation and vaccine efficacy.[58] Parasite proteins displayed during the parasitic cycle (mainly extracellular phase and cell invasion) are promising vaccine candidates.[59] The annotation of the Hungarian *B. canis* CHIPZ genome and subsequent prediction of the *B. canis* exportome provides a framework for potential vaccine target candidates. This includes proteins from the Bc28 protein family, which are highly expressed at the merozoite surface[30] and are also present during virulent infection. Naturally acquired antibody response targeting particularly the merozoite surface has a protective effect in human malaria suggesting that the recombinant proteins may be efficient vaccine candidates.[60]

In the absence of stable *in vitro* culturing systems for *B. canis*, *in silico* prediction of the parasite-host interactome could further assist in the screening for protein candidates. Mainly two different computational approaches have been described: i) Homology and structure based approaches and ii) domain and motif interaction based approaches, which both include *in silico* machine training/learning and data mining.[61] Nevertheless, all current prediction approaches lack biological evidence and result in high false positive rates and are not reliable to replace biophysical approaches and animal experiments.[62] A further approach for high throughput immunological screening of protein candidates are cell-free expression systems, which represent simple and efficient protein production platforms.[63-65] They are based on linear or plasmid based DNA-templates applicable in multi-well format allowing to by-pass multiple steps, which are typically required using conventional *in vivo* protein expression systems. In summary, the herein provided data forms the backbone for future improvements in fighting *B. canis* including parasites own proteomic targets.

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## Curriculum Vitae


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| 09/2002 – 11/2007 | Studies of veterinary medicine, Vetsuisse Faculty, UZH (Dipl. med. vet.) |
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Armua-Fernandez M.T., Joekel D., Schweiger A., **Eichenberger R.M.**, Matsumoto J., Deplazes P. (2016). Successful intestinal *Echinococcus multilocularis* oncosphere invasion and subsequent hepatic metacestode establishment in resistant RccHan™:WIST rats after pharmacological immunosuppression. *Parasitology.*

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**Eichenberger R.M.**, Karvountzis S., Ziadinov I., Deplazes P. (2011). Severe *Taenia ovis* outbreak in a sheep flock in south-west England. *Veterinary Record* 168 (23), 619

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Eichenberger R.M., Strabel D., Deplazes P. (2011). Bandwurmfinnen beim Rind. *UFA-Revue* 5, 43-6

Eichenberger R.M., Strabel D., Deplazes P. (2011). Cysticerques du ver solitaire. *UFA-Revue* 5, 43-6

Eichenberger R.M., Deplazes P. (2009). Braucht es neue Bekämpfungsstrategien gegen den Rinderfinnenbefall? *Schweizer Zoonosebericht 2008*, 12-15

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WAAVP, Liverpool, UK, 2015: "Prognostic markers for the outcome of acute *Babesia canis* infections"

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